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APPLICATION NUMBER: *60/504,428*
FILING DATE: *September 22, 2003*
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Certified by

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Attorney Docket No. 41860-193139

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
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☐ *Additional inventors are being named on the _____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (280 characters max)

ANTIGENS FOR AN EAST COAST FEVER VACCINE

CORRESPONDENCE ADDRESS

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* 84 ☐ CD(s), Number

☒ Drawing(s) *Number of Sheets* 21 ☒ Other (specify) Annex 7 pages

☒ Application Data Sheet. See 37 CFR 1.76

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☒ Applicant claims small entity status. See 37 CFR 1.27.

☐ A check or money order is enclosed to cover the filing fees

FILING FEE AMOUNT ($)

☒ The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 22-0261 80.00

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____.

Respectfully submitted,
SIGNATURE Nancy Axelrod, Reg No 44,014 for
Date 09/22/03
REGISTRATION NO. *(if appropriate)* 31,957
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USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. and is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C. 20231.

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☒ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 80

Complete If Known	
Application Number	To be assigned
Filing Date	September 22, 2003
First Named Inventor	Evans TARACHA
Examiner Name	To be assigned
Group / Art Unit	To be assigned
Attorney Docket No.	41860-19313

METHOD OF PAYMENT (check all that apply)

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Deposit Account Number	22-0261
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FEE CALCULATION

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1001	750	2001	375	Utility filing fee	
1002	330	2002	165	Design filing fee	
1003	520	2003	260	Plant filing fee	
1004	750	2004	375	Reissue filing fee	
1005	160	2005	80	Provisional filing fee	80

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2. EXTRA CLAIM FEES

			Extra Claims		Fee from below	=	Fee Paid
Total Claims		- ** =	0	X		=	0
Independent Claims		- ** =	0	X		=	0
Multiple Dependent				X		=	0

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
1202	18	2202	9	Claims in excess of 20
1201	84	2201	42	Independent claims in excess of 3
1203	280	2203	140	Multiple dependent claim, if not paid
1204	84	2204	42	** Reissue independent claims over original patent
1205	18	2205	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($) 0

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FEE CALCULATION (continued)

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1051	130	2051	65	Surcharge - late filing fee or oath	
1052	50	2052	25	Surcharge - late provisional filing fee or cover sheet.	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing request for ex parte reexam	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	410	2252	205	Extension for reply within second month	
1253	930	2253	465	Extension for reply within third month	
1254	1,450	2254	725	Extension for reply within fourth month	
1255	1,970	2255	985	Extension for reply within fifth month	
1401	320	2401	160	Notice of Appeal	
1402	320	2402	160	Filing a brief in support of an appeal	
1403	280	2403	140	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive – unavoidable	
1453	1,300	2453	650	Petition to revive – unintentional	
1501	1,300	2501	650	Utility issue fee (or reissue)	
1502	470	2502	235	Design issue fee	
1503	630	2503	315	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17 (q)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	750	2809	375	Filing a submission after final rejection (37 CFR § 1.129(a))	
1810	750	2810	375	For each additional invention to be examined (37 CFR § 1.129(b))	
1801	750	2801	375	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	
				Other fee (specify) Re	

*Reduced by Basic Filing Fee Paid SUBTOTAL (3) ($) 0

SUBMITTED BY				Complete *(if applicable)*	
Name (Print/Type)	Nancy J. Axelrod	*Registration No. Attorney/Agent)*	44,014	*Telephone*	(202) 344-8334
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PC Docs No. 486131

ANTIGENS FOR AN EAST COAST FEVER VACCINE

BACKGROUND OF THE INVENTION

Theilerioses are a group of disease syndromes affecting cattle, sheep, goats and domestic buffalo caused by tick-borne haemo-protozoan parasites in the genus *Theileria*. The most economically important diseases include Mediterranean fever, East Coast fever (ECF) and Malignant theileriosis. Mediterranean fever caused by *Theileria annulata* occurs in North Africa, southern Europe, Near East, Middle East and many parts of Far East Asia with a population of 200 million cattle and buffalo at risk. ECF, caused by *T. parva*, affects 30 million cattle in eastern, central and southern Africa. Malignant theileriosis caused by *T. lestoquardi* affects sheep and goats in southeastern Europe, North Africa, the Near and Middle East and southern Russia and neighboring States. These parasites belong to the same api-complexan group as *Plasmodium falciparum*, *Toxoplasma gondii*, *Cytoxauzoon spp*, *Eimeria spp* and *Babesia spp*, with a life-cycle having the arthropod and mammalian components in which sexual and asexual stages develop, respectively. The pathogenic stages of *Theileria* parasites differ. *T. parva* causes a lympho-proliferative disorder in which schizont-infected lymphoblasts are responsible for the pathogenesis of the disease. On the other hand, anemic disease caused by *T. lestoquardi* and *T. sergenti* is due to piroplasm-infected erythrocytes while both the schizont and piroplasm of *T. annulata* are pathogenic resulting in lympho-proliferative and anemic syndromes, respectively.

Currently, theilerioses are controlled largely by tick control using acaricides and through "infection and treatment" vaccination protocols, of animals at risk. Due to cost and problems of tick resistance and environmental pollution, control of these diseases through acaricidal destruction of ticks is not sustainable. Vaccination, on the other hand, while effective presents with certain shortcomings associated with the use of live vaccines. Owing to the ease with which to transmit *T. annulata*, infected blood was originally used to immunize cattle with parasites of low virulence but were still accompanied by clinical episodes. With the advent of *in vitro* cultivation of *T. annulata* (Shama et al., 1998) and the development of bulk culture techniques in the 1960s, significant progress was made in realizing a practical immunization strategy. Currently, passage-attenuated cultures of *T. annulata* are routinely used in national vaccination programs in affected countries. By contrast, similar efforts to immunize cattle against *T. parva* were unsuccessful. This was attributed to the failure of attenuated *T. parva* parasites to induce immunity. In addition, much higher numbers of *T. parva*-infected cells were required to infect cattle reliably since the schizonts of *T. parva* transfer at a low frequency and donor cells get rejected before successful transfer. *T. lestoquardi* has also been cultivated *in vitro* and studies have shown that attenuated parasites can be used to immunize animals with a degree of success.

Given the unsuccessful attempts to immunize cattle with attenuated *T. parva*, subsequent efforts have focused on the use of virulent parasites with accompanying chemotherapy. The rationale of this infection and treatment method (ITM) is to allow the infection to establish and suppress development of patent clinical disease by administering theileriacidal drugs. Animals thus immunized were found to be protected against the homologous parasite. This vaccination strategy has undergone successive refinement including the use of cryopreserved triturated tick stabilites containing sporozoites (the parasite stage infective for cattle lymphocytes) to standardize the infection dose, as well as simultaneous drug administration. Further improvement of this immunization approach has involved the identification and use a combination of parasite stocks to broaden the immunizing spectrum of the vaccine against several field *T. parva* parasite populations. But the use of local parasite stocks to immunize in areas where they have been isolated is also practiced. ITM immunization against *T. parva* has been tested extensively under laboratory and field conditions and is now deployed in the affected region to control ECF.

ITM is a very efficacious vaccine; however, it has a number of practical limitations that hinder its application as a sustainable control measure against ECF. Being live, it requires a cold or refrigeration chain, which is impractical in Africa, and also causes clinical disease if drug application is inadequate and has the potential to introduce new parasite strains in areas under the vaccination campaign. The cost (US$10-20 per immunization) this vaccine is well beyond the poor farmers afflicted by ECF due to the cost of the drugs and the requirement for a trained veterinarian to administer the vaccine. Because of these concerns for the ITM vaccine, a great deal of investment has been put in research work to develop a vaccine that will be sustainable.

Antigens of parasitic protozoans that induce a protective antibody response against the development of disease have been identified. For example, the major merozoite surface protein of Plasmodium species has been shown to be a target of varying degrees of protective immunity against the asexual blood stages in rodent and human malaria. Vaccination of mice with purified P230, the major merozoite surface protein of the rodent malaria *Plasmodium yoelii*, has resulted in reduced parasitemias in comparison to controls upon intravenous challenge with a lethal dose of parasitized erythrocytes (Holder et al. 1981. Nature 294:361). Mice have also been protected against P. yoelii by passive transfer of a monoclonal antibody (Mab) specific for P230 (Majarian et al. 1984. J. Immunol. 132:3131) and against (rodent malaria) *Plasmodium chabaudi adami* challenge by passive immunization with a Mab specific for the homologous 250-kDa molecule of this *Plasmodium* species (Lew et al. 1989. Proc. Natl. Acad. Sci. USA 86:3768).

A 67 kDa glycoprotein (p67) from the surface of the *T. parva* sporozoite has been isolated (U.S. Patent Number 5273744). Cattle recovering from a single infection with *T. parva* sporozoites resist homologous challenge. These animals have weak

antibody and T cell responses to p67. However, when repeatedly exposed to sporozoites, high anti-p67 antibodies are detected which neutralize infectivity of sporozoites. Cattle trials with this candidate vaccine have demonstrated less than optimal protection despite induction of strong antibody responses using traditional antigen delivery methods. Protection engendered following a single infection has been shown to be based on the induction of class I MHC-restricted $CD8^+$ cytotoxic T lymphocytes (CTLs) whose target parasite antigens would be prime vaccine candidates. These studies, conducted over a period of 20 years, have made the development of an improved sub-unit vaccine against ECF a reasonable probability. However, it has been impossible to identify the specific pathogen antigens that could induce a $CD8^+$ cytotoxic T lymphocyte response. Methods using traditional reagents and cell lines have failed to identify particular antigens that will stimulate this CD8+ lymphocyte pathway.

SUMMARY OF THE INVENTION

The present invention relates to compositions and methods for the identification of parasite antigens, such as *Theileria parva* antigens, that trigger antigen-specific cytotoxic T lymphocyte (CTLs) responses, for inducing immunoprotection against *T. parva* in bovine species. The compositions include proteins, polypeptides, antigens, epitopes and nucleic acids that encode proteins, polypeptides, epitopes and antigens. More particularly, the method steps wherein the stimulation of responding lymphocytes to cells transfected by cDNAs encoding parasite antigen, is measured in a high throughput manner, by the release of soluble factors, such as gamma interferon, using either an antibody-elispot assay or a bioassay employing endothelial cells; the use of immortalized skin fibroblast cells from outbred animals, that have recovered from exposure, as antigen presenting cells, enables antigen identification, especially, where cloned bovine MHC class I genes are not available for co-transfection into COS cells; and/or the resolution of the identity of individual antigens from candidate cDNA pools.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graph of Elispot data, indicating detection of BoLa class I restricted CTL responses to schizont infected cells.

FIG. 2 is a graph of Elispot data, indicating the sensitivity of IFN-gamma Elispot to detect CTL recognition of schizont infected cells.

FIG. 3 is a graph of Elispot data, indicating the effects of CTL age, post-stimulation upon responses to schizont infected cells as detected by IFN-gamma Elispot.

FIG. 4 is a graph indicating the effect of reduced Tp1 and Tp2 DNA for transfection of COS and iSF upon recognition by schizont specific BV115 and BWo14 polyclonal CD8+ CTL lines, respectively.

FIG. 5 is a graph indicating the recognition of selected gene number 5 transfected iSF by BW002, BW014, CD8+ polyclonal CTL lines and D409 CTL clone number 10.

FIG. 6 is a graph indicating the % lysis of BW002 and BV050 cells, transfected with a cDNA for Tp2.

FIG. 7 is a graph indicating the % lysis of BW002 and BV050 cells, transfected with a cDNA for Tp2, by the schizont-specific BW014 polyclonal CD8+ CTL line.

FIG. 8 is a graph indicating the % lysis of BW002 and BV050 cells, transfected with a cDNA for Tp2, by w7 restricted schizont specific D409 CTL clone number 10.

FIG. 9 is a graph, indicating the mapping of Tp2 CTL epitopes using synthetic peptides.

FIG. 10 is a graph, indicating the mapping of Tp2 CTL epitopes using synthetic 9-mer peptides.

FIG. 11 is a graph indicating the lysis of Tp2 synthetic peptide pulsed autologous iSF by the schizont specific D409 CTL clone number 10.

FIG. 12 is a graph indicating the lysis of Tp2 synthetic peptide pulsed autologous iSF by the schizont specific BW014 polyclonal CD8+ CTL line.

FIG. 13 is a series of graphs indicating the Tp2 specific CD8+ T cell responses following challenge of immune cattle.

FIG. 14 is a graph indicating the lysis of Tp2 peptide pulsed autologous iSF by TpM stimulated PBMC.

FIG. 15 is a photograph of a Coomassie stained gel and an anti-His-tag immunoblot (Western blot) of Tp2 expression, using a bacterial expression vector..

FIG. 16 is a picture of a gel, demonstrating the expression of recombinant Tp3.

FIG. 17 is a picture of a gel, demonstrating the expression of recombinant Tp6.

FIG. 18 is the DNA sequence and deduced protein sequence of Tp2.

FIG. 19 is the DNA sequence and deduced protein sequence of Tp3.

FIG. 20 is the DNA sequence and deduced protein sequence of Tp6.

FIG 21 is the DNA sequence and deduced protein sequence of Tp2 epitopes 1, 2, 3, and 4.

DETAILED DESCRIPTION OF THE INVENTION

Class I MHC-restricted $CD8^+$ cytotoxic T lymphocytes (CTLs) are responsible for protecting cattle against a lethal challenge with *T. parva* sporozoites (several reviews cited). These CTLs are directed at schizont-infected cells, which they recognize- and lyse. Schizont antigens that are recognized by these $CD8^+$ CTLs are the prime candidates for inclusion into an effective sub-unit vaccine for ECF.

The present invention provides polynucleotides, and methods for their identification, which encode useful proteins. The useful proteins encoded by the polynucleotide sequences of the subject invention are targets of antigen-specific cytotoxic CD8+ T lymphocytes which have been shown to be protective in adoptive cell transfer experiments.

Antigens encoded by polynucleotide sequences, cloned from candidate expressed genes of *Theileria parva*, are identified by conventional nucleic acid sequence alignment and parsing programs in which conditions for the searching include signal peptide sequences, anchor motifs and homology to known CTL (CD8+) are used. These are confirmed to be stimulating antigens with the use of immortalized cloned bovine skin fibroblast cell lines. In another preferred embodiment, stimulation of cytotoxic CD8+ T lymphocytes, by antigen, is measured by the release of soluble factors, more specifically gamma interferon. The subject invention also relates to compositions isolated by methods including one or more of these particular steps and the use of these compositions: to stimulate or induce cytotoxic T cells, as diagnostic reagents for the detection of disease, or an immune response, in kits or high throughput "chip" methods for the detection of or expression of, identical or homologous nucleic acids.

As a result of the degeneracy of the genetic code, a multitude of nucleotide sequences may be produced which are based upon the sequences provided herein and corresponding peptides, polypeptides, or proteins. Some of these nucleotide sequences will bear only minimal homology to the sequences disclosed herein; however the subject invention specifically contemplates each and every possible variation of nucleotide sequence that could be made by selecting combinations based on possible codon choices. These combinations are made in accordance with the standard triplet genetic code as applied to the nucleotide sequence of naturally occurring peptide, polypeptide, or protein, and all such variations are to be considered as being specifically disclosed herein.

It is possible to produce the polynucleotides of the subject invention, or portions thereof, entirely by synthetic chemistry. After synthesis, the nucleic acid sequence can be used alone or joined with a preexisting sequence and inserted into one of the many available DNA vectors and their respective host cells using techniques well known in the art. Moreover, synthetic chemistry may be used to introduce specific mutations into the nucleotide sequence. Alternatively, a portion of sequence in which a mutation is desired can be synthesized and recombined with a portion of an existing genomic or recombinant sequence.

Nucleotide sequences encoding a peptide, polypeptide, or protein may be joined to a variety of other nucleotide sequences by means of well established recombinant DNA techniques (Sambrook J. et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.; or Ausubel F. M. et al. (1989) Current Protocols in Molecular Biology, John Wiley & Sons, New York City). Useful sequences include an assortment of cloning vectors such as plasmids, cosmids, lambda phage derivatives, phagemids, and the like. Vectors of interest include vectors for replication, expression, probe generation, sequencing, and the like. In general, vectors of interest may contain an origin of replication functional in at least one organism, convenient restriction endonuclease sensitive sites, and selectable markers for one or more host cell systems.

Another aspect of the subject invention is to provide for hybridization probes which are capable of hybridizing with naturally occurring antigen sequences or nucleotide sequences encoding the disclosed peptide, polypeptide, or protein. The stringency of the hybridization conditions will determine whether the probe identifies only the native nucleotide sequence or sequences of closely related molecules. If degenerate nucleotide sequences of the subject invention are used for the detection of related sequences, they should preferably contain at least 50% of the nucleotides of the sequences presented herein.

Hybridization probes of the subject invention may be derived from the nucleotide sequences of the attached List Sequences and the Sequences provided in FIGS. 16-20, or from surrounding or included genomic sequences comprising untranslated regions such as promoters, enhancers and introns. Such hybridization probes may be labeled with appropriate reporter molecules. Means for producing specific hybridization probes include oligolabelling, nick translation, end-labeling or PCR amplification using a labeled nucleotide. Alternatively, the cDNA sequence may be cloned into a vector for the production of mRNA probe. Such vectors are known in the art, are commercially available, and may be used to synthesize RNA probes in vitro by addition of an appropriate RNA polymerase such as T7, T3 or SP6 and labeled nucleotides. A number of companies (such as Pharmacia Biotech, Piscataway, N.J.; Promega, Madison, Wis.; US Biochemical Corp, Cleveland, Ohio; etc.) supply commercial kits and protocols for these procedures.

The nucleotide sequences (shown in FIGS. 16-20,) can be used to generate probes for mapping the native genomic sequence. The sequence may be mapped to a particular chromosome or to a specific region of the chromosome using well known techniques. These include in situ hybridization to chromosomal spreads, flow-sorted chromosomal preparations, or artificial chromosome constructions such as yeast artificial chromosomes (YACs), bacterial artificial chromosomes (BACs), bacterial P1 constructions or single chromosome cDNA libraries.

In situ hybridization of chromosomal preparations and physical mapping techniques such as linkage analysis using established chromosomal markers are invaluable in extending genetic maps. The nucleotide sequences of the subject invention may also be used to detect differences in the chromosomal location of nucleotide sequences due to translocation, inversion, or recombination.

Other aspects of the invention include use of the disclosed sequences or recombinant nucleic acids derived there from to produce purified peptides. The nucleotide sequences as disclosed herein may be used to produce an amino acid sequence using well known methods of recombinant DNA technology. Goeddel (Gene Expression Technology, Methods and Enzymology [1990] Vol 185, Academic Press, San Diego, Calif.) is one among many publications which teach expression of an isolated, purified nucleotide sequence. The amino acid or peptide may be expressed in a variety of host cells, either prokaryotic or eukaryotic. Host cells may be from the same species from which the nucleotide sequence was derived or from a different species.

Still further aspects of the invention use these purified peptides to produce antibodies or other molecules able to bind to the peptides. These antibodies or binding agents can then be used for the screening of cells in order to localize the cellular distribution of the peptides or proteins. The antibodies are also useful for the affinity purification of recombinantly produced peptides or proteins. Such antibodies are also useful as diagnostic reagents for the detection of protozoan diseases or in antibody-mediated tests and assays.

Additionally, the proteins, polypeptides, fragments, and peptides that represent antigenic epitopes described in the present disclosure, can be employed to stimulate the immune response of a mammal in order to induce an antibody production response (B-cell), as well as a stimulatory or protective T-cell response. In addition, in vitro assays have shown that the proteins, fragments, polypeptides, and epitopes of the present antigens can stimulate the production of soluble immune factors such as interferon, both in tissue cell culture situations as well as in an intact mammal. The present proteins, fragments, polypeptides, and peptides can stimulate the immune system of a variety of mammals, e.g., mice, goats, and bovines. Antibodies to these present proteins can be detected in the serum of mammals or in the tissue culture supernatant of tissue culture stimulation situations.

the two complementary strands. These bonds can be broken by treatments such as high pH or high temperature, and these conditions result in the dissociation, or "denaturation," of the two strands. If the DNA is then placed in conditions which make hydrogen bonding of the bases thermodynamically favorable, the DNA strands will anneal, or "hybridize," and reform the original double-stranded DNA. If carried out under appropriate conditions, this hybridization can be highly specific. That is, only strands with a high degree of base complementarity will be able to form stable double-stranded structures. The relationship of the specificity of hybridization to reaction conditions is well known. Thus, hybridization may be used to test whether two pieces of DNA are complementary in their base sequences. It is this hybridization mechanism which facilitates the use of probes of the subject invention to readily detect and characterize DNA sequences of interest.

The specifically exemplified polynucleotides of the subject invention can themselves be used as probes. Additional polynucleotide sequences can be added to the ends of (or internally in) the exemplified polynucleotide sequences so that polynucleotides that are longer than the exemplified polynucleotides can also be used as probes. Thus, isolated polynucleotides comprising one or more of the exemplified sequences are within the scope of the subject invention. Polynucleotides that have less nucleotides than the exemplified polynucleotides can also be used and are contemplated within the scope of the present invention. For example, for some purposes, it might be useful to use a conserved sequence from an exemplified polynucleotide wherein the conserved sequence comprises a portion of an exemplified sequence. Thus, polynucleotides of the subject invention can be used to find additional, homologous (wholly or partially) genes.

Probes of the subject invention may be composed of DNA, RNA, or PNA (peptide nucleic acid). The probe will normally have at least about 10 bases, more usually at least about 17 bases, and may have up to about 100 bases or more. Longer probes can readily be utilized, and such probes can be, for example, several kilobases in length. The probe sequence is designed to be at least substantially complementary to a portion of a gene encoding a protein of interest. The probe need not have perfect complementarity to the sequence to which it hybridizes. The probes may be labeled utilizing techniques that are well known to those skilled in this art.

One approach for the use of the subject invention as probes entails first identifying DNA segments that are homologous with the disclosed nucleotide sequences using, for example, Southern blot analysis of a gene bank. Thus, it is possible, without the aid of biological analysis, to know in advance the probable activity of many new polynucleotides, and of the individual gene products expressed by a given polynucleotide. Such an analysis provides a rapid method for identifying commercially valuable compositions.

One hybridization procedure useful according to the subject invention typically

T. parva is known to cause a lymphoproliferative response in infected animals that resembles a variety of T-cell lymphomas, leukemias and other lymphocytic cancers. The present T. parva proteins could have utility in formulating an anti-cancer composition, e.g., antigenic proteins could have utility in prevention schemes, whereby radioactively labeled-antigens would compete for intracellular or extracellular binding sites in order to destroy cells undergoing lymphoproliferation.

Alternatively, the T. parva polypeptides of the invention can be used as a basis for identifying (screening for) agents that modify cell proliferation and/or cell transformation. Some of the T. parva polypeptides of the invention are related to (*e.g.*, have orthologues to) polypeptides of other species, such as humans. Therefore, identification of an agent that modulates (*e.g.*, inhibits) the ability of a T. parva polypeptide of interest to stimulate cell proliferation and/or to induce or maintain a transformed phenotype, in infected animals or in cell culture, could also provide an agent that inhibits cell proliferation in an animal, such as a cow, goat or human, *e.g.*, that acts as an anti-neoplastic agent. Among the types of agents that could be screened with such an assay are small molecules, for example small molecule libraries obtained by combinatorial chemistry, or libraries of natural products; antibodies; or the like.

Alternatively, in some cases an antibody specific for a polypeptide of the invention is also specific for an orthologous protein, such as a human protein. In this case, an antibody of the invention (specific for a T. parva polypeptide of the invention) can be used to deliver a toxic agent to a cell, such as human cell, expressing the orthologous polypeptide, *e.g.*, to act as a "magic bullet." Such a magic bullet could be used to treat cancers, such as, *e.g.*, leukemias, in a human. Agents that can be conjugated to, and delivered by, such an antibody are well-known to those of skill in the art.

The disclosed nucleotide sequences can be used individually, or in panels, in tests or assays to detect levels of peptide, polypeptide, or protein expression. The form of such qualitative or quantitative methods may include northern analysis, dot blot or other membrane based technologies, dip stick, pin or chip technologies, PCR, ELISAs or other multiple sample format technologies.

Polynucleotide probes employing the present inventive compositions. DNA possesses a fundamental property called base complementarity. In nature, DNA ordinarily exists in the form of pairs of anti-parallel strands, the bases on each strand projecting from that strand toward the opposite strand. The base adenine (A) on one stand will always be opposed to the base thymine (T) on the other strand, and the base guanine (G) will be opposed to the base cytosine (C). The bases are held it apposition by their ability to hydrogen bond in this specific way. Though each individual bond is relatively weak, the net effect of many adjacent hydrogen bonded bases, together with base stacking effects, is a stable joining of

includes the initial steps of isolating the DNA sample of interest and purifying it chemically. Either lysed cells or total fractionated nucleic acid isolated from cells can be used. Cells can be treated using known techniques to liberate their DNA (and/or RNA). The DNA sample can be cut into pieces with an appropriate restriction enzyme. The pieces can be interest can be through electrophoresis in a gel, usually agarose or acrylamide. The pieces of interest can be transferred to an immobilizing membrane.

The particular hybridization technique is not essential to the subject invention. As improvements are made in hybridization techniques, they can be readily applied.

The probe and sample can then be combined in a hybridization buffer solution and held at an appropriate temperature until annealing occurs. Thereafter, the membrane is washed free of extraneous materials, leaving the sample and bound probe molecules typically detected and quantified by autoradiography and/or liquid scintillation counting. As is well known in the art, if the probe molecule and nucleic acid sample hybridize by forming a strong non-covalent bond between the two molecules, it can be reasonably assumed that the probe and sample are essentially identical or very similar. The probe's detectable label provides a means for determining in a known manner whether hybridization has occurred.

In the use of the nucleotide segments as probes, the particular probe is labeled with any suitable label known to those skilled in the art, including radioactive and non-radioactive labels. Typical radioactive labels include ^{32}P, ^{35}S, or the like that can be detected using well-known methods, e.g., autoradiography. Non-radioactive labels include, for example, ligands such as biotin or thyroxine that can be detected using labeled molecules –for example, biotin can be detected using phycoerythrin-labeled avidin, as well as enzymes such as hydrolases or peroxidases, or the various chemiluminescent molecules such as luciferin, or fluorescent compounds like fluorescein and its derivatives. In addition, the probes can be made inherently fluorescent as described in International Application No. WO 93/16094. DNA and RNA can be chemically labeled by exploiting the inherent reactivity of these molecules. For example, the cytidine residues can be directly labeled with biotin using a bisulfate coupling reaction. The presence of doubled stranded nucleic acids can also be detected by laser light interrogation for the binding of DNA-staining fluorescent dyes such as propidum iodide (PI) and Hoechst 33342 (HO).

Various degrees of stringency of hybridization can be employed. The more stringent the conditions, the greater the complementarity that is required for duplex formation. The degree of complementarity is an indication of the amount of specificity, e.g. the amount of non-covalent interaction of complementary of sequence betweens two DNA/RNA nucleic acid strands or molecules, e.g. under very stringent conditions, a run of sequence of '3-AAATTCGGG-5' would specifically interact strongly with a complementary strand of '5-TTTAAGCCC-3'.

While under less-stringent conditions it would be possible for a lot of mismatching, yet at the same time keeping a high degree of interaction between the two strands. Stringency can be controlled by temperature, probe concentration, probe length, ionic strength, time, and the like. Preferably, hybridization is conducted under moderate to high stringency conditions by techniques well known in the art, as described, for example, in Keller, G. H., M. M. Manak (1987) DNA Probes, Stockton Press, New York, N.Y., pp. 169-170.

As used herein "moderate to high stringency" conditions for hybridization refers to conditions that achieve the same, or about the same, degree of specificity of hybridization as the conditions "as described herein." Examples of moderate to high stringency conditions are provided herein. Specifically, hybridization of immobilized DNA on Southern blots with .sup.32P-labeled gene-specific probes is performed using standard methods (Maniatis et al.). In general, hybridization and subsequent washes are carried out under moderate to high stringency conditions that allow for detection of target sequences with homology to sequences exemplified herein. For double-stranded DNA gene probes, hybridization is carried out overnight at 20-25.degree. C. below the melting temperature (Tm) of the DNA hybrid in 6.times. SSPE, 5.times. Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. The melting temperature is described by the following formula from Beltz et al. (1983):

Tm=81.5.degree. C.+16.6 Log[Na+]+0.41(% G+C)-0.61(% formamide)-600/length of duplex in base pairs.

Washes are typically carried out as follows:

(1) Twice at room temperature for 15 minutes in 1.times. SSPE, 0.1% SDS (low stringency wash).

(2) Once at Tm-20.degree. C. for 15 minutes in 0.2.times. SSPE, 0.1% SDS (moderate stringency wash).

For oligonucleotide probes, hybridization is carried out overnight at 10-20.degree. C. below the melting temperature (Tm) of the hybrid in 6.times. SSPE, 5.times. Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. Tm for oligonucleotide probes is determined by the following formula from Suggs et al. (1981):

Tm (.degree. C.)=2(number T/A base pairs)+4(number G/C base pairs)

Washes are typically carried out as follows:

(1) Twice at room temperature for 15 minutes 1.times. SSPE, 0.1% SDS (low stringency wash).

(2) Once at the hybridization temperature for 15 minutes in 1.times. SSPE, 0.1% SDS (moderate stringency wash).

In general, salt and/or temperature can be altered to change stringency. With a labeled DNA fragment of greater than about 70 or so bases in length, the following conditions can be used:

1 Low: 1 or 2X SSPE, room temperature Low: 1 or 2X SSPE, 42.degree. C. Moderate: 0.2X or 1X SSPE, 65.degree. C. High: 0.1X SSPE, 65.degree. C.

Duplex formation and stability depend on substantial complementarity between the two strands of a hybrid, and, as noted above, a certain degree of mismatch can be tolerated. Therefore, polynucleotide sequences of the subject invention include mutations (both single and multiple), deletions, and insertions in the described sequences, and combinations thereof, wherein said mutations, insertions, and deletions permit formation of stable hybrids with a target polynucleotide of interest. Mutations, insertions, and deletions can be produced in a given polynucleotide sequence using standard methods known in the art. Other methods may become known in the future.

The mutational, insertional, and deletional variants of the polypeptide sequences of the invention can be used in the same manner as the exemplified polynucleotide sequences so long as the variants have substantial sequence similarity with the original sequence. As used herein, substantial sequence similarity refers to the extent of nucleotide similarity that is sufficient to enable the variant polynucleotide to function in the same capacity as the original sequence. Preferably, this similarity is greater than 50%; more preferably, this similarity is greater than 75%; and most preferably, this similarity is greater than 90%. The degree of similarity needed for the variant to function in its intended capacity will depend upon the intended use of the sequence. It is well within the skill of a person trained in this art to make mutational, insertional, and deletional mutations that are designed to improve the function of the sequence or otherwise provide a methodological advantage. As relates to the present invention, all variants are considered to be active variants, wherein the functional activity of stimulating T cell lymphocytes and eliciting protective immunity against an antigen such as *T. parva* is retained by the variant and extends to all variants that exhibit this functional activity. In the case of fragments, the range of acceptable fragment sizes extends from full length to the epitope level, e.g. from a sequences size of a protein with a chain length of 500 amino acids down to a peptide length of 8-9 amino acids. It is not unusual for T cell epitopes to be in the size range of 6-10 amino acids, with such an epitope retaining functional activity as described above with regard to cells of the immune response.

PCR technology. Polymerase Chain Reaction (PCR) is a repetitive, enzymatic,

primed synthesis of a nucleic acid sequence. This procedure is well known and commonly used by those skilled in this art (see U.S. Pat. Nos. 4,683,195, 4,683,202, and 4,800,159; Saiki et al., 1985). PCR is based on the enzymatic amplification of a DNA fragment of interest that is flanked by two oligonucleotide primers that hybridize to opposite strands of the target sequence. The primers are oriented with the 3' ends pointing towards each other. Repeated cycles of heat denaturation of the template, annealing of the primers to their complementary sequences, and extension of the annealed primers with a DNA polymerase result in the amplification of the segment defined by the 5' ends of the PCR primers. Since the extension product of each primer can serve as a template for the other primer, each cycle essentially doubles the amount of DNA fragment produced in the previous cycle. This results in the exponential accumulation of the specific target fragment, up to several million-fold in a few hours. By using a thermostable DNA polymerase such as Taq polymerase, which is isolated from the thermophilic bacterium Thermus aquaticus, the amplification process can be completely automated. Other enzymes that can be used are known to those skilled in the art.

The polynucleotide sequences of the subject invention (and portions thereof such as conserved regions and portions that serve to distinguish these sequences from previously-known sequences) can be used as, and/or used in the design of, primers for PCR amplification. In performing PCR amplification, a certain degree of mismatch can be tolerated between primer and template. Therefore, mutations, deletions, and insertions (especially additions of nucleotides to the 5' end) of the exemplified polynucleotides can be used in this manner. Mutations, insertions and deletions can be produced in a given primer by methods known to an ordinarily skilled artisan.

Full length genes may be cloned utilizing partial nucleotide sequence and various methods known in the art. Gobinda et al. (1993; PCR Methods Applic 2:318-22) disclose "restriction-site PCR" as a direct method which uses universal primers to retrieve unknown sequence adjacent to a known locus. First, genomic DNA is amplified in the presence of primer to linker and a primer specific to the known region. The amplified sequences are subjected to a second round of PCR with the same linker primer and another specific primer internal to the first one. Products of each round of PCR are transcribed with an appropriate RNA polymerase and sequenced using reverse transcriptase.

Inverse PCR can be used to acquire unknown sequences starting with primers based on a known region (Triglia T. et al. (1988) Nucleic Acids Res 16:8186). The method uses several restriction enzymes to generate a suitable fragment in the known region of a gene. The fragment is then circularized by intramolecular ligation and used as a PCR template. Divergent primers are designed from the known region. The multiple rounds of restriction enzyme digestions and ligations that are necessary prior to PCR make the procedure slow and expensive (Gobinda et al. [1993] supra).

Capture PCR (Lagerstrom M. et al. (1991) PCR Methods Applic 1:111-19) is a method for PCR amplification of DNA fragments adjacent to a known sequence in eukaryotic and YAC DNA. As noted by Gobinda et al. (1993,supra), capture PCR also requires multiple restriction enzyme digestions and ligations to place an engineered double-stranded sequence into an unknown portion of the DNA molecule before PCR. Although the restriction and ligation reactions are carried out simultaneously, the requirements for extension, immobilization and two rounds of PCR and purification prior to sequencing render the method cumbersome and time consuming.

Parker J. D. et al. (Nucleic Acids Res [1991 119:3055-60), teach walking PCR, a method for targeted gene walking which permits retrieval of unknown sequences. PromoterFinder.TM. is a kit available from Clontech Laboratories, Inc. (Palo Alto, Calif.) which uses PCR and primers derived from p53 to walk in genomic DNA. Nested primers and special PromoterFinder.TM. Libraries are used to detect upstream sequences such as promoters and regulatory elements. This process avoids the need to screen libraries and is useful in finding intron/exon junctions.

One PCR method replaces methods which use labeled probes to screen plasmid libraries and allow one researcher to process only about 3-5 genes in 14-40 days. In the first step, which can be performed in about two days, any two of a plurality of primers are designed and synthesized based on a known partial sequence. In step 2, which takes about six to eight hours, the sequence is extended by PCR amplification of a selected library. Steps 3 and 4, which take about one day, are purification of the amplified cDNA and its ligation into an appropriate vector. Step 5, which takes about one day, involves transforming and growing up host bacteria. In step 6, which takes approximately five hours, PCR is used to screen bacterial clones for extended sequence. The final steps, which take about one day, involve the preparation and sequencing of selected clones.

If the full length cDNA has not been obtained, the entire procedure is repeated using either the original library or some other preferred library. The preferred library may be one that has been size-selected to include only larger cDNAs or may consist of single or combined commercially available libraries, e.g., from Clontech Laboratories, Inc. (Palo Alto, Calif.). The cDNA library may have been prepared with oligo (dT) or random priming. Random primed libraries are preferred in that they will contain more sequences which contain 5' ends of genes. A randomly primed library may be particularly useful if an oligo (dT) library does not yield a complete gene. It must be noted that the larger and more complex the protein, the less likely it is that the complete gene will be found in a single plasmid.

CLONTECH PCR-Select.TM. cDNA Subtraction (Clontech Laboratories, Inc., Palo Alto, Calif.) is yet another means by which differentially expressed genes may be isolated. The procedure allows for the isolation of transcripts present in one mRNA

population which is absent, or found in reduced numbers, in a second population of mRNA. Rare transcripts may be enriched 1000-fold.

Another method for analyzing either the size or the nucleotide sequence of PCR products is capillary electrophoresis. Systems for rapid sequencing are available from Perkin Elmer (Foster City Calif.), Beckman Instruments (Fullerton, Calif.), and other companies. Capillary sequencing employs flowable polymers for electrophoretic separation, four different fluorescent dyes (one for each nucleotide) which are laser activated, and detection of the emitted wavelengths by a charge coupled devise camera. Output/light intensity is converted to electrical signal using appropriate software (e.g. Genotyper.TM. and Sequence Navigators.TM. from Perkin Elmer) and the entire process from loading of samples to computer analysis and electronic data display is computer controlled. Capillary electrophoresis provides greater resolution and is many times faster than standard gel based procedures. It is particularly suited to the sequencing of small pieces of DNA which might be present in limited amounts in a particular sample. The reproducible sequencing of up to 350 bp of M13 phage DNA in 30 min has been reported (Ruiz-Martinez M. C. et al. [1993] Anal Chem 65:2851-8).

Polynucleotides and proteins. Polynucleotides of the subject invention can be defined according to several parameters. One characteristic is the biological activity of the protein products as identified herein. The proteins and genes of the subject invention can be further defined by their amino acid and nucleotide sequences. The sequences of the molecules can be defined in terms of homology to certain exemplified sequences as well as in terms of the ability to hybridize with, or be amplified by, certain exemplified probes and primers. Additional primers and probes can readily be constructed by those skilled in the art such that alternate polynucleotide sequences encoding the same amino acid sequences can be used to identify and/or characterize additional genes. The proteins of the subject invention can also be identified based on their immunoreactivity with certain antibodies.

The polynucleotides and proteins of the subject invention include portions, fragments, variants, and mutants of the full-length sequences as well as fusions and chimerics, so long as the encoded protein retains the characteristic biological activity of the proteins identified herein. As used herein, the terms "variants" or "variations" of genes refer to nucleotide sequences that encode the same proteins or which encode equivalent proteins having equivalent biological activity. As used herein, the term "equivalent proteins" refers to proteins having the same or essentially the same biological activity as the exemplified proteins.

Variations of genes may be readily constructed using standard techniques such as site-directed mutagenesis and other methods of making point mutations and by DNA shuffling, for example. In addition, gene and protein fragments can be made using commercially available exonucleases, endonucleases, and proteases

according to standard procedures. For example, enzymes such as Bal31 can be used to systematically cut off nucleotides from the ends of genes. Also, genes that encode fragments may be obtained using a variety of restriction enzymes. Proteases may be used to directly obtain active fragments of these proteins. Of course, molecular techniques for cloning polynucleotides and producing gene constructs of interest are also well known in the art. In vitro evaluation techniques, such as MAXYGEN's "Molecular Breeding" can also be applied to practice the subject invention.

Because of the redundancy of the genetic code, a variety of different DNA sequences can encode the amino acid sequences encoded by the polynucleotide sequences disclosed herein. It is well within the skill of a person trained in the art to create these alternative DNA sequences encoding proteins having the same, or essentially the same, amino acid sequence. These variant DNA sequences are within the scope of the subject invention. As used herein, reference to "essentially the same" sequence refers to sequences that have amino acid substitutions, deletions, additions, or insertions that do not materially affect biological activity. Fragments retaining the characteristic biological activity are also included in this definition.

A farther method for identifying genes and polynucleotides (and the proteins encoded thereby) of the subject invention is through the use of oligonucleotide probes. Probes provide a rapid method for identifying genes of the subject invention. The nucleotide segments that are used as probes according to the invention can be synthesized using a DNA synthesizer and standard procedures.

The subject invention comprises variant or equivalent proteins (and nucleotide sequences coding for equivalent proteins) having the same or similar biological activity of proteins encoded by the exemplified polynucleotides. Equivalent proteins will have amino acid similarity with an exemplified protein (or peptide). The amino acid identity will typically be greater than 60%. Preferably, the amino acid identity will be greater than 75%. More preferably, the amino acid identity will be greater than 80%, and even more preferably greater than 90%. Most preferably, amino acid identity will be greater than 95%. (Likewise, the polynucleotides that encode the subject polypeptides will also have corresponding identities in these preferred ranges.) These identities are as determined using standard alignment techniques for determining amino acid identity. The amino acid identity/similarity/homology will be highest in critical regions of the protein including those regions that account for biological activity or that are involved in the determination of three-dimensional configuration that is ultimately responsible for the biological activity. In this regard, certain amino acid substitutions are acceptable and can be expected if these substitutions are in regions which are not critical to activity or are conservative amino acid substitutions which do not affect the three-dimensional configuration of the molecule. For example, amino acids may be placed in the following classes: non-polar, uncharged polar, basic, and

acidic. Conservative substitutions whereby an amino acid of one class is replaced with another amino acid of the same type fall within the scope of the subject invention so long as the substitution does not materially alter the biological activity of the compound. Below is a list of examples of amino acids belonging to various classes.

Class of Amino Acid Examples of Amino Acids Nonpolar Ala, Val, Leu, Ile, Pro, Met, Phe, Trp Uncharged Polar Gly, Ser, Thr, Cys, Tyr, Asn, Gln Acidic Asp, Glu Basic Lys, His

In some instances, non-conservative substitutions can also be made.

As used herein, reference to "isolated" polynucleotides and/or "purified" proteins refers to these molecules when they are not associated with the other molecules with which they would be found in nature. Thus, reference to "isolated" and/or "purified" signifies the involvement of the "hand of man" as described herein. Reference to "heterologous" proteins, genes, and gene constructs, also signifies the involvement of the "hand of man."

Vectors/Host Cells

The invention also provides vectors containing the nucleic acid molecules described herein. The term "vector" refers to a vehicle, preferably a nucleic acid molecule, which can transport the nucleic acid molecules. When the vector is a nucleic acid molecule, the nucleic acid molecules are covalently linked to the vector nucleic acid. With this aspect of the invention, the vector includes a plasmid, single or double stranded phage, a single or double stranded RNA or DNA viral vector, or artificial chromosome, such as a BAC, PAC, YAC, OR MAC.

A vector can be maintained in the host cell as an extrachromosomal element where it replicates and produces additional copies of the nucleic acid molecules. Alternatively, the vector may integrate into the host cell genome and produce additional copies of the nucleic acid molecules when the host cell replicates.

The invention provides vectors for the maintenance (cloning vectors) or vectors for expression (expression vectors) of the nucleic acid molecules. The vectors can function in prokaryotic or eukaryotic cells or in both (shuttle vectors).

Expression vectors contain cis-acting regulatory regions that are operatively linked in the vector to the nucleic acid molecules such that transcription of the nucleic acid molecules is allowed in a host cell. All types of regulatory regions that affect the transcription or expression of nuclei acid molecules are also called expression control sequences. The nucleic acid molecules can be introduced into the host cell with a separate nucleic acid molecule capable of affecting transcription. Thus, the second nucleic acid molecule may provide a trans-acting factor interacting with

the cis-regulatory control region to allow transcription of the nucleic acid molecules from the vector. Alternatively, a trans-acting factor may be supplied by the host cell. Finally, a trans-acting factor can be produced from the vector itself. It is understood, however, that in some embodiments, transcription and/or translation of the nucleic acid molecules can occur in a cell-free system.

The regulatory sequence to which the nucleic acid molecules described herein can be operatively linked include promoters for directing mRNA transcription. These include, but are not limited to, the left promoter from bacteriophage .lambda., the lac, TRP, and TAC promoters from E. Coli, the early and late promoters from SV40, the CMV immediate early promoter, the adenovirus early and late promoters, and retrovirus long-terminal repeats.

In addition to control regions that promote transcription, expression vectors may also include regions that modulate transcription, such as repressor binding sites and enhancers. Examples include the SV40 enhancer, the cytomegalovirus immediate early enhancer, polyoma enhancer, adenovirus enhancers, and retrovirus LTR enhancers.

In addition to containing sites for transcription initiation and control, expression vectors can also contain sequences necessary for transcription termination and, in the transcribed region a ribosome binding site for translation. Other regulatory control elements for expression include initiation and termination codons as well as polyadenylation signals. The person of ordinary skill in the art would be aware of the numerous regulatory sequences that are useful in expression vectors. Such regulatory sequences are described, for example, in Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd. ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (1989).

A variety of expression vectors can be used to express a nucleic acid molecule. Such vectors include chromosomal, episomal, and virus-derived vectors, for example vectors derived from bacterial plasmids, from bacteriophage, from yeast episomes, from yeast chromosomal elements, including yeast artificial chromosomes, from viruses such as baculoviruses, papovaviruses such as SV40, Vaccinia viruses, adenoviruses, poxviruses, pseudorabies viruses, and retroviruses. Vectors may also be derived from combinations of these sources such as those derived from plasmid and bacteriophage genetic elements, e.g. cosmids and phagemids. Appropriate cloning and expression vectors for prokaryotic and eukaryotic hosts are described in Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd. ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (1989).

The regulatory sequence may provide constitutive expression in one or more host cells (i.e. tissue specific) or may provide for inducible expression in one or more cell types such as by temperature, nutrient additive, or exogenous factor such as a

hormone or other ligand. A variety of vectors providing for constitutive and inducible expression in prokaryotic and eukaryotic hosts are well known to those of ordinary skill in the art.

The nucleic acid molecules can be inserted into the vector nucleic acid by well-known methodology. Generally, the DNA sequence that will ultimately be expressed is joined to an expression vector by cleaving the DNA sequence and the expression vector with one or more restriction enzymes and then ligating the fragments together. Procedures for restriction enzyme digestion and ligation are well known to those of ordinary skill in the art.

The vector containing the appropriate nucleic acid molecule can be introduced into an appropriate host cell for propagation or expression using well-known techniques. Bacterial cells include, but are not limited to, E. coli, Streptomyces, and Salmonella typhimurium. Eukaryotic cells include, but are not limited to, yeast, insect cells such as Drosophila, animal cells such as COS and CHO cells, and plant cells.

As described herein, it may be desirable to express the peptide as a fusion protein. Accordingly, the invention provides fusion vectors that allow for the production of the peptides. Fusion vectors can increase the expression of a recombinant protein, increase the solubility of the recombinant protein, and aid in the purification of the protein by acting for example as a ligand for affinity purification. A proteolytic cleavage site may be introduced at the junction of the fusion moiety so that the desired peptide can ultimately be separated from the fusion moiety. Proteolytic enzymes include, but are not limited to, factor Xa, thrombin, and enteroenzyme. Typical fusion expression vectors include pGEX (Smith et al., Gene 67:31-40 (1988)), pMAL (New England Biolabs, Beverly, Mass.) and pRIT5 (Pharmacia, Piscataway, N.J.) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein. Examples of suitable inducible non-fusion E. coli expression vectors include pTrc (Amann et al, Gene 69:301-315 (1988)) and pET 11d (Studier et al, Gene Expression Technology: Methods in Enzymology 185:60-89 (1990)).

Recombinant protein expression can be maximized in host bacteria by providing a genetic background wherein the host cell has an impaired capacity to proteolytically cleave the recombinant protein. (Gottesman, S., Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, Calif. (1990) 119-128). Alternatively, the sequence of the nucleic acid molecule of interest can be altered to provide preferential codon usage for a specific host cell, for example E. coli. (Wada et al., Nucleic Acids Res. 20:2111-2118 (1992)).

The nucleic acid molecules can also be expressed by expression vectors that are operative in yeast. Examples of vectors for expression in yeast e.g., S. cerevisiae

include pYepSecI (Baldari, et al, EMBO J. 6:229-234 (1987)), pMFa (Kujan et al, Cell 30:933-943(1982)), pJRY88 (Schultz et al, Gene 54:113-123 (1987)), and pYES2 (Invitrogen Corporation, San Diego, Calf.).

The nucleic acid molecules can also be expressed in insect cells using, for example, baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (e.g., Sf9 cells) include the pAc series (Smith et al, Mol. Cell Biol. 3:2156-2165 (1983)) and the pVL series (Lucklow et al, Virology 170:31-39 (1989)).

In certain embodiments of the invention, the nucleic acid molecules described herein are expressed in mammalian cells using mammalian expression vectors. Examples of mammalian expression vectors include pCDM8 (Seed, B. Nature 329:840(1987)) and pMT2PC (Kaufman et al., EMBO J. 6:187-195 (1987)).

The expression vectors listed herein are provided by way of example only of the well-known vectors available to those of ordinary skill in the art that would be useful to express the nucleic acid molecules. The person of ordinary skill in the art would be aware of other vectors suitable for maintenance propagation or expression of the nucleic acid molecules described herein. These are found for example in Sambrook, J., Fritsh, E. F., and Maniatis, T. Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989.

The invention also encompasses vectors in which the nucleic acid sequences described herein are cloned into the vector in reverse orientation, but operatively linked to a regulatory sequence that permits transcription of antisense RNA. Thus, an antisense transcript can be produced to all, or to a portion, of the nucleic acid molecule sequences described herein, including both coding and non-coding regions. Expression of this antisense RNA is subject to each of the parameters described above in relation to expression of the sense RNA (regulatory sequences, constitutive or inducible expression, tissue-specific expression).

The invention also relates to recombinant host cells containing the vectors described herein. Host cells therefore include prokaryotic cells, lower eukaryotic cells such as yeast, other eukaryotic cells such as insect cells, and higher eukaryotic cells such as mammalian cells.

The recombinant host cells are prepared by introducing the vector constructs described herein into the cells by techniques readily available to the person of ordinary skill in the art. These include, but are not limited to, calcium phosphate transfection, DEAE-dextran-mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection, lipofection, and other techniques such as those found in Sambrook, et al. (Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor

Laboratory Press, Cold Spring Harbor, N.Y., 1989).

Host cells can contain more than one vector. Thus, different nucleotide sequences can be introduced on different vectors of the same cell. Similarly, the nucleic acid molecules can be introduced either alone or with other nucleic acid molecules that are not related to the nucleic acid molecules such as those providing trans-acting factors for expression vectors. When more than one vector is introduced into a cell, the vectors can be introduced independently, co-introduced or joined to the nucleic acid molecule vector.

In the case of bacteriophage and viral vectors, these can be introduced into cells as packaged or encapsulated virus by standard procedures for infection and transduction. Viral vectors can be replication-competent or replication-defective. In the case in which viral replication is defective, replication will occur in host cells providing functions that complement the defects.

Vectors generally include selectable markers that enable the selection of the subpopulation of cells that contain the recombinant vector constructs. The marker can be contained in the same vector that contains the nucleic acid molecules described herein or may be on a separate vector. Markers include tetracycline or ampicillin-resistance genes for prokaryotic host cells and dihydrofolate reductase or neomycin resistance for eukaryotic host cells. However, any marker that provides selection for a phenotypic trait will be effective.

While the mature proteins can be produced in bacteria, yeast, mammalian cells, and other cells under the control of the appropriate regulatory sequences, cell-free transcription and translation systems can also be used to produce these proteins using RNA derived from the DNA constructs described herein.

Where secretion of the peptide is desired, which is difficult to achieve with multi-transmembrane domain containing proteins such as MHC Class I-binding peptides, appropriate secretion signals are incorporated into the vector. The signal sequence can be endogenous to the peptides or heterologous to these peptides.

The expressed protein can be isolated from the host cell by standard disruption procedures, including freeze thaw, sonication, mechanical disruption, use of lysing agents and the like. The peptide can then be recovered and purified by well-known purification methods including ammonium sulfate precipitation, acid extraction, anion or cationic exchange chromatography, phosphocellulose chromatography, hydrophobic-interaction chromatography, affinity chromatography, hydroxylapatite chromatography, lectin chromatography, or high performance liquid chromatography.

It is also understood that depending upon the host cell in recombinant production of the peptides described herein, the peptides can have various glycosylation

patterns, depending upon the cell, or maybe non-glycosylated as when produced in bacteria. In addition, the peptides may include an initial modified methionine in some cases as a result of a host-mediated process.

There are many methods for introducing a heterologous gene or polynucleotide into a host cell or cells under conditions that allow for stable maintenance and expression of the gene or polynucleotide. These methods are well known to those skilled in the art. Examples would include infection-viral vector-mediated transfer, e.g., adenovirus, retroviral vectors, lentiviruses; physical methods such as electroporation, particle bombardment, DNA injection, and gene-gun methods; combinations of these techniques such as electroporation and lipofection, and the like. Synthetic genes, such as, for example, those genes modified to enhance expression in a heterologous host (such as by preferred codon usage or by the use of adjoining, downstream, or upstream enhancers) that are functionally equivalent to the genes (and which encode equivalent proteins) can also be used to transfect hosts. Methods for the production of synthetic genes are known in the art. Recombinant hosts can be used for the expression or propagation of genes and polynucleotides of the present invention. The genes and polynucleotides within the scope of the present invention can be introduced into a wide variety of microbial or plant hosts, such as bacterial cells, yeast, insect cells, plant cell cultures or plants, mammalian cells. For example, T. parva nucleic acids can be expressed in a recombinant baculovirus (BV) possessing an optimized promoter and translation initiation region operatively linked to the T. parva nucleic acid. In a preferred embodiment, an optimized promoter and translation initiation region are operably linked to the T. parva nucleic acid. In one embodiment, insect host cells can be transformed with baculovirus expressing T. parva nucleic acids. In still another embodiment, Tn5 (Trichoplusia ni or High Five.TM.) host cells can be transformed with baculovirus expressing T. parva nucleic acids.

T. parva "recombinant protein", is a protein made using recombinant techniques, i.e. through the expression of a recombinant nucleic acid as depicted above. A recombinant protein is distinguished from naturally occurring protein by at least one or more characteristics. For example, the protein may be isolated or purified away from some or all of the proteins and compounds with which it is normally associated in its wild type host, and thus may be substantially pure. For example, an isolated protein is unaccompanied by at least some of the material with which it is normally associated in its natural state, preferably constituting at least about 0.5%, more preferably at least about 5% by weight of the total protein in a given sample. A substantially pure protein comprises at least about 75% by weight of the total protein, with at least about 80% being preferred, and at least about 90% being particularly preferred. The definition includes the production of a protein from one organism in a different organism or host cell. Alternatively, the protein may be made at a significantly higher concentration than is normally seen, through the use of an inducible promoter or high expression promoter, such that the protein is made at increased concentration levels. Alternatively, the protein may be in a form not normally found in nature, as in the addition of an epitope tag or amino acid

substitutions, insertions and/or deletions, as discussed below.

Included in the definition of T. parva antigen polypeptides are T. parva polypeptide variants. These variants fall into one or more of three classes: substitutional, insertional or deletional variants. These variants ordinarily are prepared by site specific mutagenesis of nucleotides in the DNA encoding a T. parva antigen polypeptide, using cassette or PCR mutagenesis, scanning mutagenesis, gene shuffling or other techniques well known in the art, to produce DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture as outlined above. However, variant T. parva polypeptide fragments having up to about 100-150 residues may be prepared by in vitro synthesis using established techniques. Amino acid sequence variants are characterized by the predetermined nature of the variation, a feature that sets them apart from naturally occurring allelic or interspecies variation of the T. parva antigen polypeptide amino acid sequence.

While the site or region for introducing an amino acid sequence variation is predetermined, the mutation per se need not be predetermined. For example, in order to optimize the performance of a mutation at a given site, random mutagenesis may be conducted at the target codon or region and the expressed T. parva antigen polypeptide variants can be screened for the optimal combination of desired activity. Techniques for making mutations at predetermined sites in DNA having a known sequence are well known. For example, the variations can be made using oligonucleotide-mediated site-directed mutagenesis [Carter et al., Nucl. Acids Res., 13:4331 (1986); Zoller et al., Nucl. Acids Res., 10:6487 (1987)], cassette mutagenesis [Wells et al., Gene, 34:315 (1985)], restriction selection mutagenesis [Wells et al., Philos. Trans. R. Soc. London SerA, 317:415 (1986)], all of which are expressly incorporated by reference, PCR mutagenesis, or other known techniques can be performed on the cloned DNA to produce T. parva antigen polypeptide variant DNA. Scanning amino acid analysis can also be employed to identify one or more amino acids along a contiguous sequence. Among the preferred scanning amino acids are relatively small, neutral amino acids. Such amino acids include alanine, glycine, serine, and cysteine. Alanine is typically a preferred scanning amino acid among this group because it eliminates the side-chain beyond the beta-carbon and is less likely to alter the main-chain conformation of the variant [Cunningham and Wells, Science, 244: 1081-1085 (1989), which is expressly incorporated by reference]. Alanine is also typically preferred because it is the most common amino acid. Further, it is frequently found in both buried and exposed positions [Creighton, The Proteins, (W.H. Freeman & Co., N.Y.); Chothia, J. Mol. Biol., 150:1 (1976), which are expressly incorporated by reference]. If alanine substitution does not yield adequate amounts of variant, an isoteric amino acid can be used. Screening of the mutants or variants is done using Elispot and/or bioassays of T. parva antigen polypeptide activities and/or properties as described herein.

The present invention further provides fragments of the antigen peptides, in addition to proteins and peptides that comprise and consist of such fragments, particularly those comprising the residues identified in FIG. 2. The fragments, to which the invention pertains, however, are not to be construed as encompassing fragments that may be disclosed publicly prior to the present invention.

As used herein, a fragment comprises at least 8, 10, 12, 14, 16, or more contiguous amino acid residues from an antigen peptide. Such fragments can be chosen based on the ability to retain one or more of the biological activities of the antigen peptide or could be chosen for the ability to perform a function, e.g. bind a substrate or act as an immunogen. Particularly important fragments are biologically active fragments, peptides that are, for example, about 8 or more amino acids in length. Such fragments will typically comprise a domain or motif of the antigen peptide, e.g., active site, a transmembrane domain or a substrate-binding domain. Further, possible fragments include, but are not limited to, domain or motif containing fragments, soluble peptide fragments, and fragments containing immunogenic structures. Predicted domains and functional sites are readily identifiable by computer programs well known and readily available to those of skill in the art (e.g., PROSITE analysis). The results of one such analysis are provided in FIG. 2.

Polypeptides often contain amino acids other than the 20 amino acids commonly referred to as the 20 naturally occurring amino acids. Further, many amino acids, including the terminal amino acids, may be modified by natural processes, such as processing and other post-translational modifications, or by chemical modification techniques well known in the art. Common modifications that occur naturally in antigen peptides are described in basic texts, detailed monographs, and the research literature, and they are well known to those of skill in the art.

Known modifications include, but are not limited to, acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent crosslinks, formation of cystine, formation of pyroglutamate, formylation, gamma carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination.

Such modifications are well known to those of skill in the art and have been described in great detail in the scientific literature. Several particularly common modifications, glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation, for instance, are described in most basic texts, such as Proteins--Structure and Molecular Properties,

2.sup.nd Ed., T. E. Creighton, W. H. Freeman and Company, New York (1993). Many detailed reviews are available on this subject, such as by Wold, F., Posttranslational Covalent Modification of Proteins, B. C. Johnson, Ed., Academic Press, New York 1-12 (1983); Seifter et al. (Meth. Enzymol. 182: 626-646 (1990)) and Rattan et al. (Ann. N.Y Acad. Sci. 663:48-62 (1992)).

Accordingly, the antigen peptides of the present invention also encompass derivatives or analogs in which a substituted amino acid residue is not one encoded by the genetic code, in which a substituent group is included, in which the mature antigen peptide is fused with another compound, such as a compound to increase the half-life of the antigen peptide (for example, polyethylene glycol), or in which the additional amino acids are fused to the mature antigen peptide, such as a leader or secretory sequence or a sequence for purification of the mature antigen peptide or a pro-protein sequence.

Antibody Production

Although an amino acid sequence or oligopeptide used for antibody induction does not require biological activity, it must be immunogenic. A peptide, polypeptide, or protein used to induce specific antibodies may have an amino acid sequence consisting of at least five amino acids and preferably at least 10 amino acids. Short stretches of amino acid sequence may be genetically or chemically fused with those of another protein such as keyhole limpet hemocyanin, and the chimeric peptide used for antibody production. Alternatively, the oligopeptide may be of sufficient length to contain an entire domain.

Antibodies specific for peptides, polypeptides, or proteins may be produced by inoculation of an appropriate animal with an antigenic fragment of the peptide, polypeptide, or protein. Antibody production includes not only the stimulation of an immune response by injection into animals, but also analogous processes such as the production of synthetic antibodies, the screening of recombinant immunoglobulin libraries for specific-binding molecules (Orlandi R. et al. [1989] PNAS 86:3833-3837, or Huse W. D. et al. [1989] Science 256:1275-1281), or the in vitro stimulation of lymphocyte populations. Current technology (Winter G. and Milstein C. [1991] Nature 349:293-299) provides for a number of highly specific binding reagents based on the principles of antibody formation. These techniques may be adapted to produce molecules which specifically bind antigen peptides. Antibodies or other appropriate molecules generated against a specific immunogenic peptide fragment or oligopeptide can be used in Western analysis, enzyme-linked immunosorbent assays (ELISA) or similar tests to establish the presence of or to quantitate amounts of peptide, polypeptide, or protein in normal, diseased, or transformed cells, tissues, organs, or organisms as well as liquid suspensions containing said peptide, polypeptide, or protein.

The invention also provides antibodies that selectively bind to one of the peptides of the present invention, a protein comprising such a peptide, as well as variants and fragments thereof. As used herein, an antibody selectively binds a target peptide when it binds the target peptide and does not significantly bind to unrelated proteins. An antibody is still considered to selectively bind a peptide even if it also binds to other proteins that are not substantially homologous with the target peptide so long as such proteins share homology with a fragment or domain of the peptide target of the antibody. In this case, it would be understood that antibody binding to the peptide is still selective despite some degree of cross-reactivity.

As used herein, an antibody is defined in terms consistent with that recognized within the art: they are multi-subunit proteins produced by a mammalian organism in response to an antigen challenge. The antibodies of the present invention include polyclonal antibodies and monoclonal antibodies, as well as fragments of such antibodies, including, but not limited to, Fab or F(ab').sub.2, and Fv fragments.

Many methods are known for generating and/or identifying antibodies to a given target peptide. Several such methods are described by Harlow, Antibodies, Cold Spring Harbor Press, (1989).

In general, to generate antibodies, an isolated peptide is used as an immunogen and is administered to a mammalian organism, such as a rat, rabbit or mouse. The full-length protein, an antigenic peptide fragment or a fusion protein can be used. Particularly important fragments are those covering functional domains, such as the domains identified in the figures, and domain of sequence homology or divergence amongst the family, such as those that can readily be identified using protein alignment methods and as presented in the Figures.

Antibodies are preferably prepared from regions or discrete fragments of the antigen proteins. Antibodies can be prepared from any region of the peptide as described herein. However, preferred regions will include those involved in function/activity and/or antigen/binding partner interaction.

An antigenic fragment will typically comprise at least 8 contiguous amino acid residues. The antigenic peptide can comprise, however, at least 10, 12, 14, 16 or more amino acid residues. Such fragments can be selected on a physical property, such as fragments correspond to regions that are located on the surface of the protein, e.g., hydrophilic regions or can be selected based on sequence uniqueness.

Detection on an antibody of the present invention can be facilitated by coupling (i.e., physically linking) the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, .beta.-galactosidase, or acetylcholinesterase; examples of suitable

prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include .sup.125I, .sup.131I, .sup.35S or .sup.3H.

The antibodies can be used to isolate one of the proteins of the present invention by standard techniques, such as affinity chromatography or immunoprecipitation. The antibodies can facilitate the purification of the natural protein from cells and recombinantly produced protein expressed in host cells. In addition, such antibodies are useful to detect the presence of one of the proteins of the present invention in cells or tissues to determine the pattern of expression of the protein among various tissues in an organism and over the course of schizont development. Such antibodies can be used to detect protein in situ, in vitro, or in a cell lysate or supernatant in order to evaluate the abundance and pattern of expression. Also, such antibodies can be used to assess abnormal tissue distribution or abnormal expression during development or progression of a protozoan infection. Antibody detection of circulating fragments of the full length protein can be used to identify turnover.

Further, the antibodies can be used to assess expression in disease states such as in active stages of the disease or in lymphocytes harvested from infected animals. Experimental data as provided for other T. parva antigens indicates expression of these antigens in bovine lymphocytes, infected with T. parva. If a disease is characterized by a specific mutation in the protein, antibodies specific for this mutant protein can be used to assay for the presence of the specific mutant protein.

The diagnostic uses can be applied, not only in genetic testing, but also in monitoring a treatment modality. Accordingly, where treatment is ultimately aimed at preventing or controlling infection, antibodies directed against the protein or relevant fragments can be used to monitor therapeutic efficacy.

The antibodies are also useful for inhibiting protein function, for example, blocking the binding of the antigenic peptide to a binding partner such as a substrate. These uses can also be applied in a therapeutic context in which treatment involves inhibiting the protein's function. An antibody can be used, for example, to block binding, thus modulating (agonizing or antagonizing) the peptides activity. Antibodies can be prepared against specific fragments containing sites required for function or against intact protein that is associated with a cell or cell membrane. See FIGS. 16-20 or 23, for structural information relating to the proteins of the present invention.

The invention also encompasses kits for using antibodies to detect the presence of a protein in a biological sample. The kit can comprise antibodies such as a labeled

or labelable antibody and a compound or agent for detecting protein in a biological sample; means for determining the amount of protein in the sample; means for comparing the amount of protein in the sample with a standard; and instructions for use. Such a kit can be supplied to detect a single protein or epitope or can be configured to detect one of a multitude of epitopes, such as in an antibody detection array. Arrays are described in detail below for nucleic acid arrays and similar methods have been developed for antibody arrays.

Nucleic Acid Molecules

The present invention further provides isolated nucleic acid molecules that encode a T. parva antigen peptide or protein of the present invention (cDNA, transcript and genomic sequence). Such nucleic acid molecules will consist of, consist essentially of, or comprise a nucleotide sequence that encodes one of the enzyme peptides of the present invention, an allelic variant thereof, or an ortholog or paralog thereof.

As used herein, an "isolated" nucleic acid molecule is one that is separated from other nucleic acid present in the natural source of the nucleic acid. Preferably, an "isolated" nucleic acid is free of sequences which naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. However, there can be some flanking nucleotide sequences, for example up to about 5KB, 4KB, 3KB, 2KB, or 1KB or less, particularly contiguous peptide encoding sequences and peptide encoding sequences within the same gene but separated by introns in the genomic sequence. The important point is that the nucleic acid is isolated from remote and unimportant flanking sequences such that it can be subjected to the specific manipulations described herein such as recombinant expression, preparation of probes and primers, and other uses specific to the nucleic acid sequences.

Moreover, an "isolated" nucleic acid molecule, such as a transcript/cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or chemical precursors or other chemicals when chemically synthesized. However, the nucleic acid molecule can be fused to other coding or regulatory sequences and still be considered isolated.

For example, recombinant DNA molecules contained in a vector are considered isolated. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or substantially) DNA molecules in solution. Isolated RNA molecules include in vivo or in vitro RNA transcripts of the isolated DNA molecules of the present invention. Isolated nucleic acid molecules according to the present invention further include such molecules produced synthetically.

Following are examples which illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight

and all solvent mixture proportions are by volume unless otherwise noted. As used herein and further defined below, "nucleic acid" may refer to either DNA or RNA, or molecules which contain both deoxy- and ribonucleotides. The nucleic acids include genomic DNA, cDNA, and oligonucleotides including sense and anti-sense nucleic acids. Such nucleic acids may also contain modifications in the ribose-phosphate backbone to increase stability and half life of such molecules in physiological environments.

The nucleic acid may be double stranded, single stranded, or contain portions of both double stranded or single stranded sequence. As will be appreciated by those in the art, the depiction of a single strand ("Watson") also defines the sequence of the other strand ("Crick"); thus the sequences depicted in the figures also include the complement of the sequence.

One method for controlling gene expression according to the subject invention provides materials that would inform the production of double-stranded interfering RNA (dsRNAi), or RNA-mediated interference (RNAi), using published methods. The terms dsRNAi and RNAi are used interchangeably herein unless otherwise noted. In a more preferred embodiment, compositions are useful for regulation of gene expression in bovine lymphocytes. Such RNA would contain a nucleotide sequence identical to a fragment of the target gene; however, RNA sequences with insertions, deletions, and point mutations relative to the target sequence can also be used for inhibition. Sequence identity may optimized by sequence comparison and alignment algorithms known in the art (see Gribskov and Devereux, Sequence Analysis Primer, Stockton Press, 1991, and references cited therein) and calculating the percent difference between the nucleotide sequences by, for example, the Smith-Waterman algorithm as implemented in the BESTFIT software program using default parameters (e.g., University of Wisconsin Genetic Computing Group). Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a fragment of the target gene transcript.

As disclosed herein, 100% sequence identity between the RNA and the target gene is not required to practice the present invention. Thus the invention has the advantage of being able to tolerate sequence variations that might be expected due to genetic mutation, strain polymorphism, or evolutionary divergence. RNAi molecules of the subject invention are not limited to those that are targeted to the full-length polynucleotide or gene. Gene product can be inhibited with an RNAi molecule that is targeted to a portion or fragment of the exemplified polynucleotides; high homology (90-95%) or greater identity is also preferred, but not necessarily essential, for such applications.

Protein/Peptide Uses

The proteins of the present invention can be used in substantial and specific assays

related to the functional information provided in the Figures; for example, to stimulate CD8+ cytotoxic T cell lines, to raise antibodies or to elicit another immune response; as a reagent (including the labeled reagent) in assays designed to quantitatively determine levels of the protein (or its binding partner or ligand) in biological fluids; and as markers for tissues in which the corresponding protein is preferentially expressed (either constitutively or at a particular stage of tissue differentiation or development or in a disease state). Where the protein binds or potentially binds to another protein or ligand (such as, for example, in an enzyme-effector protein interaction or enzyme-ligand interaction), the protein can be used to identify the binding partner/ligand so as to develop a system to identify inhibitors of the binding interaction. Any or all of these uses are capable of being developed into reagent grade or kit format for commercialization as commercial products.

Methods for performing the uses listed above are well known to those skilled in the art. References disclosing such methods include "Molecular Cloning: A Laboratory Manual", 2d ed., Cold Spring Harbor Laboratory Press, Sambrook, J., E. F. Fritsch and T. Maniatis eds., 1989, and "Methods in Enzymology: Guide to Molecular Cloning Techniques", Academic Press, Berger, S. L. and A. R. Kimmel eds., 1987.

The potential uses of the peptides of the present invention are based primarily on the source of the protein as well as the class/action of the protein. For example, antigens isolated from T. parva and their protozoan orthologs serve as targets for identifying agents for use in mammalian therapeutic applications, e.g. an animal drug, particularly in modulating a biological or pathological response in a cell, tissue, or protozoan, which expresses an immunogenic antigen. Experimental data as provided in FIG. 23 indicates that the antigens of the present invention are expressed in infected host lymphocytes, as indicated by flow cytometric analysis. Such uses can readily be determined using the information provided herein, that which is known in the art, and routine experimentation.

The proteins of the present invention are also useful in drug screening assays, in cell-based or cell-free systems. Cell-based systems can be native, i.e., cells that normally express the enzyme, as a biopsy or expanded in cell culture. Experimental data as provided in FIG. 22 indicates expression in infected bovine lymphocytes. In an alternate embodiment, cell-based assays involve recombinant host cells expressing the antigen protein.

The polypeptides can be used to identify compounds that modulate pathogen activity as measured by the antigen in its natural state or an altered form that causes a specific disease or pathology associated with the protozoan. Both the antigens of the present invention and appropriate variants and fragments can be used in high-throughput screens to assay candidate compounds for the ability to bind to the antigen. These compounds can be further screened against a functional T-cell antigen to determine the effect of the compound on the enzyme activity.

Further, these compounds can be tested in animal or invertebrate systems to determine activity/effectiveness.

The invention further includes other end point assays to identify compounds that modulate (stimulate or inhibit) protozoan antigen expression.

The proteins of the present invention are also useful in competition binding assays in methods designed to discover compounds that interact with the T. parva antigens (e.g. binding partners and/or ligands). Thus, a compound is exposed to an antigen polypeptide under conditions that allow the compound to bind or to otherwise interact with the polypeptide. Soluble antigen polypeptide is also added to the mixture. If the test compound interacts with the soluble antigen polypeptide, it decreases the amount of complex formed or activity from the antigen target. This type of assay is particularly useful in cases in which compounds are sought that interact with specific regions of the antigen. Thus, the soluble polypeptide that competes with the target antigen region is designed to contain peptide sequences corresponding to the region of interest.

To perform cell free drug screening assays, it is sometimes desirable to immobilize either the antigen protein, or fragment, or its target molecule to facilitate separation of complexes from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay.

Techniques for immobilizing proteins on matrices can be used in the drug screening assays. In one embodiment, a fusion protein can be, provided which adds a domain that allows the protein to be bound to a matrix. For example, glutathione-S-transferase fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, Mo.) or glutathione derivatized microtitre plates, which are then combined with the cell lysates (e.g., ^{35}S-labeled) and the candidate compound, and the mixture incubated under conditions conducive to complex formation (e.g., at physiological conditions for salt and pH). Following incubation, the beads are washed to remove any unbound label, and the matrix immobilized and radiolabel determined directly, or in the supernatant after the complexes are dissociated. Alternatively, the complexes can be dissociated from the matrix, separated by SDS-PAGE, and the level of antigen-binding protein found in the bead fraction quantitated from the gel using standard electrophoretic techniques. For example, either the polypeptide or its target molecule can be immobilized utilizing conjugation of biotin and streptavidin using techniques well known in the art. Alternatively, antibodies reactive with the protein but which do not interfere with binding of the protein to its target molecule can be derivatized to the wells of the plate, and the protein trapped in the wells by antibody conjugation. Preparations of an antigen-binding protein and a candidate compound are incubated in the antigen protein-presenting wells and the amount of complex trapped in the well can be quantitated. Methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes

using antibodies reactive with the antigen protein target molecule, or which are reactive with antigen protein and compete with the target molecule, as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the presence of the target molecule.

Agents that modulate one of the antigens of the present invention can be identified using one or more of the above assays, alone or in combination. It is generally preferable to use a cell-based or cell free system first and then confirm activity in an animal or other model system. Such model systems are well known in the art and can readily be employed in this context.

In yet another aspect of the invention, the antigen proteins can be used as "bait proteins" in a two-hybrid assay or three-hybrid assay (see, e.g., U.S. Pat. No. 5,283,317; Zervos et al. (1993) Cell 72:223-232; Madura et al. (1993) J. Biol. Chem. 268:12046-12054; Bartel et al. (1993) Biotechniques 14:920-924; Iwabuchi et al. (1993) Oncogene 8:1693-1696; and Brent WO94/10300), to identify other proteins, which bind to or interact with the antigen and are involved in T-cell induction activity. Such antigen-binding proteins are also likely to be involved in the propagation of signals by antigen-binding proteins or antigen targets as, for example, downstream elements of a MHC Class I-mediated signaling pathway. Alternatively, such antigen-binding proteins could act in a manner as to be inhibitors of an immune response.

This invention further pertains to novel agents identified by the above-described screening assays. Accordingly, it is within the scope of this invention to further use an agent identified as described herein in an appropriate animal model. For example, an agent identified as described herein (e.g., an antigen-modulating agent, an antisense antigen nucleic acid molecule, or an antigen-specific antibody, can be used in an animal or other model to determine the efficacy, toxicity, or side effects of treatment with such an agent. Alternatively, an agent identified as described herein can be used in an animal or other model to determine the mechanism of action of such an agent. Furthermore, this invention pertains to uses of novel agents identified by the above-described screening assays for treatments as described herein.

The antigen proteins of the present invention are also useful to provide a target for diagnosing a disease or a disease mediated by the peptide. Accordingly, the invention provides methods for detecting the presence, or levels of, the protein (or encoding mRNA) in a cell, tissue, or organism. The method involves contacting a biological sample with a compound capable of interacting with the antigen protein such that the interaction can be detected. Such an assay can be provided in a single detection format or a multi-detection format such as an antibody chip array. In addition, the proteins, polynucleotides, fragments (of the Tp2, Tp3 and Tp6 proteins) could be utilized directly as probes, both in a liquid format, e.g. binding of

antibodies present in the serum of immunized of infected animals or, --in a solid-substrate format, wherein the binding of antibody present in the serum of an infected or immunized animal to an immobilized Tp2 polypeptide, peptide, epitope, or fragment could be used as an assay to detect the presence of an antibody or a T-Cell mediated binding.

One agent for detecting a protein in a sample is an antibody capable of selectively binding to protein. This type of binding is highly specific, in that the antibody-antigen interaction is a high affinity binding reaction, e.g. an equilibrium dissociation constant of 2.6×10^{-10} M, or apparent Hill coefficient of 1.3. A biological sample includes tissues, cells and biological fluids isolated from a subject, as well as tissues, cells and fluids present within a subject.

The peptides of the present invention also provide targets for diagnosing active parasite activity or disease, in an animal having a variant peptide, particularly activities and conditions that are known for other members of the family of proteins to which the present one belongs. Thus, the peptide can be isolated from a biological sample and assayed for the presence of a genetic mutation that results in aberrant peptide. This includes amino acid substitution, deletion, insertion, rearrangement, (as the result of aberrant splicing events), and inappropriate post-translational modification. Analytic methods include altered electrophoretic mobility, altered tryptic peptide digest, altered enzyme activity in cell-based or cell-free assay, alteration in substrate or antibody-binding pattern, altered isoelectric point, direct amino acid sequencing, and any other of the known assay techniques useful for detecting mutations in a protein. Such an assay can be provided in a single detection format or a multi-detection format such as an antibody chip array.

In vitro techniques for detection of peptide include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations and immunofluorescence using a detection reagent, such as an antibody or protein binding agent, as well as the methods that represent embodiments of the present invention. Alternatively, the peptide can be detected in vivo in a subject by introducing into the subject a labeled anti-peptide antibody or other types of detection agent. For example, the antibody can be labeled with a radioactive marker whose presence and location in a subject can be detected by standard imaging techniques. Such methods can be used to detect the allelic variant of a peptide expressed in an infected subject and methods which detect fragments of a peptide in a sample.

The diagnostic uses can be applied, not only in genetic testing, but also in monitoring a treatment modality. Accordingly, where treatment is ultimately aimed at preventing or controlling infection, antibodies directed against the protein or relevant fragments can be used to monitor therapeutic efficacy.

The antibodies are also useful for inhibiting protein function, for example, blocking the binding of the antigenic peptide to a binding partner such as a substrate. These

uses can also be applied in a therapeutic context in which treatment involves inhibiting the protein's function. An antibody can be used, for example, to block binding, thus modulating (agonizing or antagonizing) the peptides activity. Antibodies can be prepared against specific fragments containing sites required for function or against intact protein that is associated with a cell or cell membrane. See FIGS. 16-20 and 23 for structural information relating to the proteins of the present invention.

The invention also encompasses kits for using antibodies to detect the presence of a protein in a biological sample. The kit can comprise antibodies such as a labeled or labelable antibody and a compound or agent for detecting protein in a biological sample; means for determining the amount of protein in the sample; means for comparing the amount of protein in the sample with a standard; and instructions for use. Such a kit can be supplied to detect a single protein or epitope or can be configured to detect one of a multitude of epitopes, such as in an antibody detection array. Arrays are described in detail below for nucleic acid arrays and similar methods have been developed for antibody arrays. There are many methods of labeling, peptides, polypeptides, antibodies and other proteins for the purpose of detecting them in a variety of assays. These are well known to those with skill in the art. Examples would include the metabolic labeling of proteins to incorporate radioisotopes such as ^{3}H and ^{35}S, that can be detected using a variety of isotope detection methods such as autoradiography; chemical labeling would include reacting the amino moieties with a radioisotope such as ^{125}I, CNBr mediated labeling with any of a variety of molecules such as biotin which can then be detected using a fluorescently labeled avidin molecule; proteins and fragments of proteins labeled with enzymes, e.g. horseradish peroxidase, provide a detection system whereby detection is measured by a color change when a substrate solution, e.g. TMB (3, 3', 5, 5'-tetramethylbenzidene) is added to the assay mixture. In addition, the presence of unlabelled antibodies could be detected by any one of a number of indirect methods that would employ antibodies directed against the anti-peptide or anti-T. parva antibodies. Such indirect methods could employ labeled anti-antibodies or labeled molecules such as lectins, bacterial proteins such as Protein A (from *Staphylococcus aureus*) or labeled *S. aureus*. A recent method, reported by Saleh and Sohn (PNAS 100:820-824, 2003) presents an electric, on-chip pore method to sense the degree of binding between antibody and antigen, wherein none of the reagents need to be labeled for a quantitative analysis of the amount of antibody or antigen present in the sample.

Nucleic Acid Molecules

The present invention further provides isolated nucleic acid molecules that encode a T. parva antigen peptide or protein of the present invention (cDNA, transcript and genomic sequence). Such nucleic acid molecules will consist of, consist essentially of, or comprise a nucleotide sequence that encodes one of the enzyme peptides of the present invention, an allelic variant thereof, or an ortholog or paralog thereof.

As used herein, an "isolated" nucleic acid molecule is one that is separated from other nucleic acid present in the natural source of the nucleic acid. Preferably, an "isolated" nucleic acid is free of sequences which naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. However, there can be some flanking nucleotide sequences, for example up to about 5KB, 4KB, 3KB, 2KB, or 1KB or less, particularly contiguous peptide encoding sequences and peptide encoding sequences within the same gene but separated by introns in the genomic sequence. The important point is that the nucleic acid is isolated from remote and unimportant flanking sequences such that it can be subjected to the specific manipulations described herein such as recombinant expression, preparation of probes and primers, and other uses specific to the nucleic acid sequences.

Moreover, an "isolated" nucleic acid molecule, such as a transcript/cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or chemical precursors or other chemicals when chemically synthesized. However, the nucleic acid molecule can be fused to other coding or regulatory sequences and still be considered isolated.

For example, recombinant DNA molecules contained in a vector are considered isolated. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or substantially) DNA molecules in solution. Isolated RNA molecules include in vivo or in vitro RNA transcripts of the isolated DNA molecules of the present invention. Isolated nucleic acid molecules according to the present invention further include such molecules produced synthetically.

Accordingly, the present invention provides nucleic acid molecules that consist of the nucleotide sequence shown in FIGS. 16-20 or any nucleic acid molecule that encodes the protein (amino acid sequence) included in these figures. A nucleic acid molecule consists of a nucleotide sequence when the nucleotide sequence is the complete nucleotide sequence of the nucleic acid molecule.

The present invention further provides nucleic acid molecules that consist essentially of the nucleotide sequence shown in FIGS. 16-20, or any nucleic acid molecule that encodes the proteins provided in the same figures. A nucleic acid molecule consists essentially of a nucleotide sequence when such a nucleotide sequence is present with only a few additional nucleic acid residues in the final nucleic acid molecule.

The present invention further provides nucleic acid molecules that comprise the nucleotide sequences shown in FIGS. 16-20, or any nucleic acid molecule that encodes the proteins provided in the same figures A nucleic acid molecule comprises a nucleotide sequence when the nucleotide sequence is at least part of the final nucleotide sequence of the nucleic acid molecule. In such a fashion, the

nucleic acid molecule can be only the nucleotide sequence or have additional nucleic acid residues, such as nucleic acid residues that are naturally associated with it or heterologous nucleotide sequences. Such a nucleic acid molecule can have a few additional nucleotides or can comprises several hundred or more additional nucleotides. A brief description of how various types of these nucleic acid molecules can be readily made/isolated is provided below.

The invention further provides nucleic acid molecules that encode fragments of the peptides of the present invention as well as nucleic acid molecules that encode obvious variants of the enzyme proteins of the present invention that are described above. Such nucleic acid molecules may be naturally occurring, such as allelic variants (same locus), paralogs (different locus), and orthologs (different organism), or may be constructed by recombinant DNA methods or by chemical synthesis. Such non-naturally occurring variants may be made by mutagenesis techniques, including those applied to nucleic acid molecules, cells, or organisms. Accordingly, as discussed above, the variants can contain nucleotide substitutions, deletions, inversions and insertions. Variation can occur in either or both the coding and non-coding regions. The variations can produce both conservative and non-conservative amino acid substitutions.

The present invention further provides epitope fragments of the antigen proteins and the nucleic acid molecules encoding the antigens, provided in the description of epitope-mapping experiments described below. An epitope coding region comprises a contiguous nucleotide sequence greater than 12 or more nucleotides. Further, a fragment could at least 30, 40, 50, 100, 250 or 500 nucleotides in length. The length of the fragment will be based on its intended use. For example, the fragment can encode epitope bearing regions of the peptide, or can be useful as DNA probes and primers. Such fragments can be isolated using the known nucleotide sequence to synthesize an oligonucleotide probe. A labeled probe can then be used to screen a cDNA library, genomic DNA library, or mRNA to isolate nucleic acid corresponding to the coding region. Further, primers can be used in PCR reactions to clone specific regions of gene.

A probe/primer typically comprises substantially a purified oligonucleotide or oligonucleotide pair. The oligonucleotide typically comprises a region of nucleotide sequence that hybridizes under stringent conditions to at least about 12, 20, 25, 40, 50 or more consecutive nucleotides.

Orthologs, homologs, and allelic variants can be identified using methods well known in the art. As described in the Peptide Section, these variants comprise a nucleotide sequence encoding a peptide that is typically 60-70%, 70-80%, 80-90%, and more typically at least about 90-95% or more homologous to the nucleotide sequence shown in the Figure sheets or a fragment of this sequence. Such nucleic

acid molecules can readily be identified as being able to hybridize under moderate to stringent conditions, to the nucleotide sequence shown in the Figure sheets or a fragment of the sequence. Allelic variants can readily be determined by genetic locus of the encoding gene. The gene encoding the novel antigen of the present invention is located on a genome component that has been mapped to the *T. parva* chromosome1, which is supported by multiple lines of evidence, such as STS and BAC map data.

Nucleic Acid Molecule Uses

The nucleic acid molecules of the present invention are useful for probes, primers, chemical intermediates, and in biological assays. The nucleic acid molecules are useful as a hybridization probe for messenger RNA, transcript/cDNA and genomic DNA to isolate full-length cDNA and genomic clones encoding the peptide described in FIG. 16-20, and to isolate cDNA and genomic clones that correspond to variants (alleles, orthologs, etc.) producing the same or related peptides shown in FIG. 16-20 and FIG 23. As illustrated in FIG. 11, deletions were identified at 2 different nucleotide positions.

The probe can correspond to any sequence along the entire length of the nucleic acid molecules provided in the Figures. Accordingly, it could be derived from 5' noncoding regions, the coding region, and 3' noncoding regions. However, as discussed, fragments are not to be construed as encompassing fragments disclosed prior to the present invention.

The nucleic acid molecules are also useful as primers for PCR to amplify any given region of a nucleic acid molecule and are useful to synthesize antisense molecules of desired length and sequence.

The nucleic acid molecules are also useful for constructing recombinant vectors. Such vectors include expression vectors that express a portion of, or all of, the peptide sequences. Vectors also include insertion vectors, used to integrate into another nucleic acid molecule sequence, such as into the cellular genome, to alter in situ expression of a gene and/or gene product. For example, an endogenous coding sequence can be replaced via homologous recombination with all or part of the coding region containing one or more specifically introduced mutations.

The nucleic acid molecules are also useful for expressing antigenic portions of the proteins.

The nucleic acid molecules are also useful as probes for determining the chromosomal positions of the nucleic acid molecules by means of in situ hybridization methods. The genes encoding the novel antigens of the present

invention are located on a genome component that has been mapped to *T. parva* chromosomes 1 (Tp2).

The nucleic acid molecules are also useful in making vectors containing the gene regulatory regions of the nucleic acid molecules of the present invention.

The nucleic acid molecules are also useful for designing ribozymes/antigens corresponding to all, or a part, of the mRNA produced from the nucleic acid molecules described herein.

The nucleic acid molecules are also useful for making vectors that express part, or all, of the peptides.

The nucleic acid molecules are also useful for constructing host cells expressing a part, or all, of the nucleic acid molecules and peptides.

The nucleic acid molecules are also useful for constructing transgenic animals expressing all, or a part, of the nucleic acid molecules and peptides.

The nucleic acid molecules are also useful as hybridization probes for determining the presence, level, form and distribution of nucleic acid expression. Such probes could be used to detect the presence of, or to determine levels of, a specific nucleic acid molecule in cells, tissues, and in organisms. The nucleic acid whose level is determined can be DNA or RNA. Accordingly, probes corresponding to the peptides described herein can be used to assess expression and/or gene copy number in a given cell, tissue, or organism. These uses are relevant for diagnosis of disorders involving an increase or decrease in antigen protein expression relative to normal results.

In vitro techniques for detection of mRNA include Northern hybridizations and in situ hybridizations. In vitro techniques for detecting DNA includes Southern hybridizations and in situ hybridization.

Probes can be used as a part of a diagnostic test kit for identifying cells or tissues that express an T. parva antigen protein, such as by measuring a level of an antigen-encoding nucleic acid in a sample of cells from a subject e.g., mRNA or genomic DNA, or determining if an antigen gene has been mutated.

Nucleic acid expression assays are useful for drug screening to identify compounds that modulate protozoan antigen nucleic acid expression.

The invention thus provides a method for identifying a compound that can be used to treat a disease associated with nucleic acid expression of the antigen gene, particularly biological and pathological processes that are mediated by the presence of T. parva in cells and tissues that express it. The method typically includes assaying the ability of the compound to modulate the expression of the antigen

nucleic acid and thus identifying a compound that can be used to treat a disorder characterized by nucleic acid expression by an infecting protozoan. The assays can be performed in cell-based and cell-free systems. Cell-based assays include cells naturally expressing the enzyme nucleic acid or recombinant cells genetically engineered to express specific nucleic acid sequences.

The assay for enzyme nucleic acid expression can involve direct assay of nucleic acid levels, such as mRNA levels, or on collateral compounds involved in an antigen-specific signal pathway. Further, the expression of genes that are up- or down-regulated in response to the antigen-stimulating signal pathway can also be assayed. In this embodiment the regulatory regions of these genes can be operably linked to a reporter gene such as luciferase.

Thus, modulators of antigen gene expression can be identified in a method wherein a cell is contacted with a candidate compound and the expression of mRNA determined. The level of expression of antigen mRNA in the presence of the candidate compound is compared to the level of expression of antigen mRNA in the absence of the candidate compound. The candidate compound can then be identified as a modulator of nucleic acid expression based on this comparison and be used, for example to treat an infection by a pathogenic protozoan. When expression of mRNA is statistically significantly greater in the presence of the candidate compound than in its absence, the candidate compound is identified as a stimulator of nucleic acid expression. When nucleic acid expression is statistically significantly less in the presence of the candidate compound than in its absence, the candidate compound is identified as an inhibitor of nucleic acid expression.

The nucleic acid molecules are also useful for monitoring the effectiveness of modulating compounds on the infectivity of a protozoan in clinical trials or in a treatment regimen. Thus, the gene expression pattern can serve as a barometer for the continuing effectiveness of treatment with the compound, particularly with compounds to which an animal or the pathogen can develop resistance. The gene expression pattern can also serve as a marker indicative of a physiological response of the affected cells to the compound.

Accordingly, such monitoring would allow either increased administration of the compound or the administration of alternative compounds to which the parasite has not become resistant. Similarly, if the level of nucleic acid expression falls below a desirable level, administration of the compound could be commensurately decreased.

The nucleic acid molecules are also useful in diagnostic assays for qualitative changes in antigen nucleic acid expression, and particularly in qualitative changes that lead to pathology. The nucleic acid molecules can be used to detect mutations in antigen genes and gene expression products such as mRNA. The nucleic acid

molecules can be used as hybridization probes to detect naturally occurring genetic mutations in the antigen gene and thereby to determine whether a parasite with the mutation is of a different strain or has a differential pathogenic potential. Mutations include deletion, addition, or substitution of one or more nucleotides in the gene, chromosomal rearrangement, such as inversion or transposition, modification of genomic DNA, such as aberrant methylation patterns or changes in gene copy number, such as amplification. Detection of a mutated form of the antigen gene associated with a dysfunction provides a diagnostic tool for an active disease or susceptibility to disease when the disease results from over-expression, under-expression, or altered expression of an antigen protein.

Parasite strains carrying mutations in the enzyme gene can be detected at the nucleic acid level by a variety of techniques. SNPs that have been found in the gene encoding the antigen of the present invention. SNPs were identified at 4 different nucleotide positions. Genomic DNA can be analyzed directly or can be amplified by using PCR prior to analysis. RNA or cDNA can be used in the same way. In some uses, detection of the mutation involves the use of a probe/primer in a polymerase chain reaction (PCR) (see, e.g. U.S. Pat. Nos. 4,683,195 and 4,683,202), such as anchor PCR or RACE PCR, or, alternatively, in a ligation chain reaction (LCR) (see, e.g., Landegran et al., Science 241:1077-1080 (1988); and Nakazawa et al., PNAS 91:360-364 (1994)), the latter of which can be particularly useful for detecting point mutations in the gene (see Abravaya et al., Nucleic Acids Res. 23:675-682 (1995)). This method can include the steps of collecting a sample of cells from an infective parasite, isolating nucleic acid (e.g., genomic, mRNA or both) from the cells of the sample, contacting the nucleic acid sample with one or more primers which specifically hybridize to a gene under conditions such that hybridization and amplification of the gene (if present) occurs, and detecting the presence or absence of an amplification product, or detecting the size of the amplification product and comparing the length to a control sample. Deletions and insertions can be detected by a change in size of the amplified product compared to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to normal RNA or antisense DNA sequences.

Alternatively, mutations in an enzyme gene can be directly identified, for example, by alterations in restriction enzyme digestion patterns determined by gel electrophoresis.

Further, sequence-specific ribozymes (U.S. Pat. No. 5,498,531) can be used to score for the presence of specific mutations by development or loss of a ribozyme cleavage site. Perfectly matched sequences can be distinguished from mismatched sequences by nuclease cleavage digestion assays or by differences in melting temperature.

Sequence changes at specific locations can also be assessed by nuclease protection assays such as RNase and S1 protection or the chemical cleavage

method. Furthermore, sequence differences between a mutant enzyme gene and a wild-type gene can be determined by direct DNA sequencing. A variety of automated sequencing procedures can be utilized when performing the diagnostic assays (Naeve, C. W., (1995) Biotechniques 19:448), including sequencing by mass spectrometry (see, e.g., PCT International Publication No. WO94/16101; Cohen et al., Adv. Chromatogr. 36:127-162 (1996); and Griffin et al., Appl. Biochem. Biotechnol. 38:147-159 (1993)).

Other methods for detecting mutations in the gene include methods in which protection from cleavage agents is used to detect mismatched bases in RNA/RNA or RNA/DNA duplexes (Myers et al., Science 230:1242 (1985)); Cotton et al., PNAS 85:4397 (1988); Saleeba et al., Meth. Enzymol. 217:286-295 (1992)), electrophoretic mobility of mutant and wild type nucleic acid is compared (Orita et al., PNAS 86:2766 (1989); Cotton et al., Mutat. Res. 285:125-144 (1993); and Hayashi et al., Genet. Anal. Tech. Appl. 9:73-79 (1992)), and movement of mutant or wild-type fragments in polyacrylamide gels containing a gradient of denaturant is assayed using denaturing gradient gel electrophoresis (Myers et al., Nature 313:495 (1985)). Examples of other techniques for detecting point mutations include selective oligonucleotide hybridization, selective amplification, and selective primer extension.

The nucleic acid molecules are also useful for testing an individual parasite for a genotype that while not necessarily causing the disease, nevertheless affects the treatment modality. Thus, the nucleic acid molecules can be used to study the relationship between a particular strain of parasite's genotype and the parasite's response to a compound used for treatment (pharmacogenomic relationship). Accordingly, the nucleic acid molecules described herein can be used to assess the mutation content of the antigen gene in an individual in order to select an appropriate compound or dosage regimen for treatment. FIG. 23 provides information on SNPs that have been found in eleven strains in the gene encoding the antigen of the present invention. SNPs were identified at nucleotide positions underlined in FIG 23. Some of these SNPs that are located outside the ORF and in introns may affect gene transcription.

Thus nucleic acid molecules displaying genetic variations that affect treatment provide a diagnostic target that can be used to tailor treatment in an individual. Accordingly, the production of recombinant cells and animals containing these polymorphisms allow effective clinical design of treatment compounds and dosage regimens.

The nucleic acid molecules are thus useful as antisense constructs to control antigen gene expression in cells, tissues, and organisms. A DNA antisense nucleic acid molecule is designed to be complementary to a region of the gene involved in transcription, preventing transcription and hence production of enzyme protein. An antisense RNA or DNA nucleic acid molecule would hybridize to the mRNA and thus

block translation of mRNA into antigen protein. Alternatively, a class of antisense molecules can be used to inactivate mRNA in order to decrease expression of antigen nucleic acid.

The invention also encompasses kits for detecting the presence of an antigen nucleic acid in a biological sample. For example, the kit can comprise reagents such as a labeled or labelable nucleic acid or agent capable of detecting antigen nucleic acid in a biological sample; means for determining the amount of antigen nucleic acid in the sample; and means for comparing the amount of antigen nucleic acid in the sample with a standard. The compound or agent can be packaged in a suitable container. The kit can further comprise instructions for using the kit to detect antigen protein mRNA or DNA.

Nucleic Acid Arrays

The present invention further provides nucleic acid detection kits, such as arrays or microarrays of nucleic acid molecules that are based on the sequence information provided in FIGS. 16-20.

As used herein "Arrays" or "Microarrays" refers to an array of distinct polynucleotides or oligonucleotides synthesized on a substrate, such as paper, nylon or other type of membrane, filter, chip, glass slide, or any other suitable solid support. In one embodiment, the microarray is prepared and used according to the methods described in U.S. Pat. No. 5,837,832, Chee et al., PCT application W095/11995 (Chee et al.), Lockhart, D. J. et al. (1996; Nat. Biotech. 14: 1675-1680) and Schena, M. et al. (1996; Proc. Natl. Acad. Sci. 93: 10614-10619), all of which are incorporated herein in their entirety by reference. In other embodiments, such arrays are produced by the methods described by Brown et al., U.S. Pat. No. 5,807,522.

The microarray or detection kit is preferably composed of a large number of unique, single-stranded nucleic acid sequences, usually either synthetic antisense oligonucleotides or fragments of cDNAs, fixed to a solid support. The oligonucleotides are preferably about 6-60 nucleotides in length, more preferably 15-30 nucleotides in length, and most preferably about 20-25 nucleotides in length. For a certain type of microarray or detection kit, it may be preferable to use oligonucleotides that are only 7-20 nucleotides in length. The microarray or detection kit may contain oligonucleotides that cover the known 5', or 3', sequence, sequential oligonucleotides which cover the full length sequence; or unique oligonucleotides selected from particular areas along the length of the sequence. Polynucleotides used in the microarray or detection kit may be oligonucleotides that are specific to a gene or genes of interest.

In order to produce oligonucleotides to a known sequence for a microarray or

detection kit, the gene(s) of interest (or an ORF identified in the present invention) is typically examined using a computer algorithm which starts at the 5' or at the 3' end of the nucleotide sequence. Typical algorithms will then identify oligomers of defined length that are unique to the gene, have a GC content within a range suitable for hybridization, and lack predicted secondary structure that may interfere with hybridization. In certain situations it may be appropriate to use pairs of oligonucleotides on a microarray or detection kit. The "pairs" will be identical, except for one nucleotide that preferably is located in the center of the sequence. The second oligonucleotide in the pair (mismatched by one) serves as a control. The number of oligonucleotide pairs may range from two to one million. The oligomers are synthesized at designated areas on a substrate using a light-directed chemical process. The substrate may be paper, nylon or other type of membrane, filter, chip, glass slide or any other suitable solid support.

In another aspect, an oligonucleotide may be synthesized on the surface of the substrate by using a chemical coupling procedure and an ink jet application apparatus, as described in PCT application W095/251116 (Baldeschweiler et al.) which is incorporated herein in its entirety by reference. In another aspect, a "gridded" array analogous to a dot (or slot) blot may be used to arrange and link cDNA fragments or oligonucleotides to the surface of a substrate using a vacuum system, thermal, UV, mechanical or chemical bonding procedures. An array, such as those described above, may be produced by hand or by using available devices (slot blot or dot blot apparatus), materials (any suitable solid support), and machines (including robotic instruments), and may contain 8, 24, 96, 384, 1536, 6144 or more oligonucleotides, or any other number between two and one million which lends itself to the efficient use of commercially available instrumentation.

In order to conduct sample analysis using a microarray or detection kit, the RNA or DNA from a biological sample is made into hybridization probes. The mRNA is isolated, and cDNA is produced and used as a template to make antisense RNA (aRNA). The aRNA is amplified in the presence of fluorescent nucleotides, and labeled probes are incubated with the microarray or detection kit so that the probe sequences hybridize to complementary oligonucleotides of the microarray or detection kit. Incubation conditions are adjusted so that hybridization occurs with precise complementary matches or with various degrees of less complementarity. After removal of nonhybridized probes, a scanner is used to determine the levels and patterns of fluorescence. The scanned images are examined to determine degree of complementarity and the relative abundance of each oligonucleotide sequence on the microarray or detection kit. The biological samples may be obtained from any bodily fluids (such as blood, urine, saliva, phlegm, gastric juices, etc.), cultured cells, biopsies, or other tissue preparations. A detection system may be used to measure the absence, presence, and amount of hybridization for all of the distinct sequences simultaneously. This data may be used for large-scale correlation studies on the sequences, expression patterns, mutations, variants, or polymorphisms among samples.

Using such arrays, the present invention provides methods to identify the expression of the antigen proteins/peptides of the present invention. In detail, such methods comprise incubating a test sample with one or more nucleic acid molecules and assaying for binding of the nucleic acid molecule with components within the test sample. Such assays will typically involve arrays comprising many genes, at least one of which is a gene of the present invention and or alleles of the enzyme gene of the present invention. The figures and associated information below provide information on epitope sequence and micro-variation in strains of T. parva that have been found in the gene encoding the antigen of the present invention.

Conditions for incubating a nucleic acid molecule with a test sample vary. Incubation conditions depend on the format employed in the assay, the detection methods employed, and the type and nature of the nucleic acid molecule used in the assay. One skilled in the art will recognize that any one of the commonly available hybridization, amplification or array assay formats can readily be adapted to employ the novel fragments of the T. parva genome disclosed herein. Examples of such assays can be found in Chard, T, An Introduction to Radioimmunoassay and Related Techniques, Elsevier Science Publishers, Amsterdam, The Netherlands (1986); Bullock, G. R. et al., Techniques in Immunocytochemistry, Academic Press, Orlando, Fla. Vol. 1 (1982), Vol. 2 (1983), Vol. 3 (1985); Tijssen, P., Practice and Theory of Enzyme Immunoassays: Laboratory Techniques in Biochemistry and Molecular Biology, Elsevier Science Publishers, Amsterdam, The Netherlands (1985).

The test samples of the present invention include cells, protein or membrane extracts of cells. The test sample used in the above-described method will vary based on the assay format, nature of the detection method and the tissues, cells or extracts used as the sample to be assayed. Methods for preparing nucleic acid extracts or of cells are well known in the art and can be readily adapted in order to obtain a sample that is compatible with the system utilized.

In another embodiment of the present invention, kits are provided which contain the necessary reagents to carry out the assays of the present invention.

Specifically, the invention provides a compartmentalized kit to receive, in close confinement, one or more containers which comprises: (a) a first container comprising one of the nucleic acid molecules that can bind to a fragment of the T. parva genome disclosed herein; and (b) one or more other containers comprising one or more of the following: wash reagents, reagents capable of detecting presence of a bound nucleic acid.

In detail, a compartmentalized kit includes any kit in which reagents are contained in separate containers. Such containers include small glass containers, plastic containers, strips of plastic, glass or paper, or arraying material such as silica. Such

containers allows one to efficiently transfer reagents from one compartment to another compartment such that the samples and reagents are not cross-contaminated, and the agents or solutions of each container can be added in a quantitative fashion from one compartment to another. Such containers will include a container which will accept the test sample, a container which contains the nucleic acid probe, containers which contain wash reagents (such as phosphate buffered saline, Tris-buffers, etc.), and containers which contain the reagents used to detect the bound probe. One skilled in the art will readily recognize that the previously unidentified enzyme gene of the present invention can be routinely identified using the sequence information disclosed herein can be readily incorporated into one of the established kit formats which are well known in the art, particularly expression arrays.

Genetically engineered host cells can be further used to produce non-human transgenic animals. A transgenic animal is preferably a mammal, for example a rodent, such as a rat or mouse, in which one or more of the cells of the animal include a transgene. A transgene is exogenous DNA which is integrated into the genome of a cell from which a transgenic animal develops and which remains in the genome of the mature animal in one or more cell types or tissues of the transgenic animal. These animals are useful for studying the function of an antigen protein and identifying and evaluating modulators of parasite development or activity. Other examples of transgenic animals include non-human primates, sheep, dogs, cows, goats, chickens, and amphibians.

A transgenic animal can be produced by introducing nucleic acid into the male pronuclei of a fertilized oocyte, e.g., by microinjection, retroviral infection, and allowing the oocyte to develop in a pseudopregnant female foster animal. Any of the enzyme protein nucleotide sequences can be introduced as a transgene into the genome of a non-human animal, such as a mouse.

Any of the regulatory or other sequences useful in expression vectors can form part of the transgenic sequence. This includes intronic sequences and polyadenylation signals, if not already included. A tissue-specific regulatory sequence(s) can be operably linked to the transgene to direct expression of the enzyme protein to particular cells.

Methods for generating transgenic animals via embryo manipulation and microinjection, particularly animals such as mice, have become conventional in the art and are described, for example, in U.S. Pat. Nos. 4,736,866 and 4,870,009, both by Leder et al., U.S. Pat. No. 4,873,191 by Wagner et al. and in Hogan, B., Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). Similar methods are used for production of other transgenic animals. A transgenic founder animal can be identified based upon the

presence of the transgene in its genome and/or expression of transgenic mRNA in tissues or cells of the animals. A transgenic founder animal can then be used to breed additional animals carrying the transgene. Moreover, transgenic animals carrying a transgene can further be bred to other transgenic animals carrying other transgenes. A transgenic animal also includes animals in which the entire animal or tissues in the animal have been produced using the homologously recombinant host cells described herein.

In another embodiment, transgenic non-human animals can be produced which contain selected systems that allow for regulated expression of the transgene. One example of such a system is the cre/loxP recombinase system of bacteriophage P1. For a description of the cre/loxP recombinase system, see, e.g., Lakso et al. PNAS 89:6232-6236 (1992). Another example of a recombinase system is the FLP recombinase system of S. cerevisiae (O'Gorman et al. Science 251:1351-1355 (1991). If a cre/loxP recombinase system is used to regulate expression of the transgene, animals containing transgenes encoding both the Cre recombinase and a selected protein is required. Such animals can be provided through the construction of "double" transgenic animals, e.g., by mating two transgenic animals, one containing a transgene encoding a selected protein and the other containing a transgene encoding a recombinase.

Clones of the non-human transgenic animals described herein can also be produced according to the methods described in Wilmut, I. et al. Nature 385:810-813 (1997) and PCT International Publication Nos. WO97/07668 and WO97/07669. In brief, a cell, e.g., a somatic cell, from the transgenic animal can be isolated and induced to exit the growth, cycle and enter G.sub.o phase. The quiescent cell can then be fused, e.g., through the use of electrical pulses, to an enucleated oocyte from an animal of the same species from which the quiescent cell is isolated. The reconstructed oocyte is then cultured such that it develops to morula or blastocyst and then transferred to pseudopregnant female foster animal. The offspring born of this female foster animal will be a clone of the animal from which the cell, e.g., the somatic cell, is isolated.

Transgenic animals containing recombinant cells that express the peptides described herein are useful to conduct the assays described herein in an in vivo context. Accordingly, the various physiological factors that are present in vivo and that could effect substrate binding, enzyme protein activation, and signal transduction, may not be evident from in vitro cell-free or cell-based assays. Accordingly, it is useful to provide non-human transgenic animals to assay in vivo enzyme protein function, including substrate interaction, the effect of specific mutant enzyme proteins on enzyme protein function and substrate interaction, and the effect of chimeric enzyme proteins. It is also possible to assess the effect of null mutations, that is, mutations that substantially or completely eliminate one or more enzyme protein functions.

The T. parva antigens of the present invention have particular potential for induction of in vivo, antigen-specific CD8+ cytotoxic T cells for prophylactic immunization of cattle for the prevention of East Coast Fever disease. In addition, CTLs specific to the following metazoan parasites may also be induced by compositions identified in accordance with the methods of the present invention: Plasmodium falciparum (which causes malaria), Schistosoma mansoni (which causes schistosomiasis), and Trypanosoma cruzi (which causes Chagas' disease), Giardia lamblia, Entoemeba histolytica, Cryptospiridium spp., Leishmania spp., Brugia spp., Wuchereria spp., Onchocerca spp., Strongyloides spp., Coccidia, Haemanchus spp., Ostertagia spp., Trichomonas spp., Dirofilaria spp., Toxocara spp., Naegleria spp., Pneumocystis carinii, Ascaris spp., other Trypanosoma spp., other Schistosome spp., other Plasmodium spp., Babesia spp., Theileria spp., including but not limited to T. parva, T. lawrencei, T. annulata, T. hirci, T. ovis, T. lastoguardi, T. orientalis, T. buffeli and T. taurotragi, Babesia spp., including, but not limited to B. bigemina, B. divergens, B. major, B. bovis, B. motasi, B. ovis, B. cabelli, B. equii, B. traumani, B. canis, B. gibsoni, B. felis, and B. microfti, Adelina app., including, but not limited to A. delina, A. castana, A. picei, A. palori, and A. triboli, Anisakis and Isospora beli. Gene alignment software indicates a high degree of homology/conservation between T. parva, and T. annulata, while T. parva and Falciparum sp. have a lesser, but significant degree of homology. Protein alignment applications, confirm this for surface antigens present in t. parva, T. annulata, and Falciparvum sp.

Vaccination strategy

Cattle trials have been initiated to assess the vaccine potential of identified CTL target antigens utilizing a recombinant canarypox virus (patented by Merial Ltd) as an antigen delivery method. The first of these trials is testing one of the candidate antigens and involves use of 16 cattle. Animals were inoculated intramuscularly with 1 ml of vaccine ($1x10^8$ pfu of virus) and boosted similarly after 4 weeks. Following a further 4 weeks, cattle will be subjected to an LD_{100} challenge with *T. parva* sporozoites by administering subcutaneously 1 ml of diluted stabilated infective material. Animals will be monitored parasitologically and clinically over a period of 2-3 weeks to determine whether the vaccine has protected.

Immunological assays are performed following immunization and challenge to evaluate antigen-specific CTL responses and relate these to the outcome to challenge.

As used herein, the following definitions apply:

An "oligonucleotide" or "oligomer" is a stretch of nucleotide residues which has a sufficient number of bases to be used in a polymerase chain reaction (PCR). These short sequences are based on (or designed from) genomic or cDNA sequences and

are used to amplify, confirm, or reveal the presence of an identical, similar or complementary DNA or RNA in a particular cell or tissue. Oligonucleotides or oligomers comprise portions of a DNA sequence having at least about 10 nucleotides and as many as about 50 nucleotides, preferably about 15 to 30 nucleotides. They can be chemically synthesized and may be used as probes.

By the term "recombinant nucleic acid" herein is meant nucleic acid, originally formed in vitro or in a cell in culture, in general, by the manipulation of nucleic acid by endonucleases and/or exonucleases and/or polymerases and/or ligases and/or recombinases, to produce a nucleic acid not normally found in nature. Thus an isolated nucleic acid, in a linear form, or an expression vector formed in vitro by ligating DNA molecules that are not normally joined, are both considered recombinant for the purposes of this invention. It is understood that once a recombinant nucleic acid is made and reintroduced into a host cell or organism, it will replicate non-recombinantly, i.e., using the in vivo cellular machinery of the host cell rather than in vitro manipulations; however, such nucleic acids, once produced recombinantly, although subsequently replicated non-recombinantly, are still considered recombinant for the purposes of the invention.

"Probes" are nucleic acid sequences of variable length, preferably between at least about 10 and as many as about 6,000 nucleotides, depending on use. They are used in the detection of identical, similar, or complementary nucleic acid sequences. Longer length probes are usually obtained from a natural or recombinant source, are highly specific and much slower to hybridize than oligomers. They may be single- or double-stranded and designed to have specificity in PCR, hybridization membrane-based, or ELISA-like technologies.

"Reporter" molecules are chemical moieties used for labeling a nucleic or amino acid sequence. They include, but are not limited to, radionuclides, enzymes, fluorescent, chemi-luminescent, or chromogenic agents. Reporter molecules associate with, establish the presence of, and may allow quantification of a particular nucleic or amino acid sequence.

A "portion" or "fragment" of a polynucleotide or nucleic acid comprises all or any part of the nucleotide sequence having fewer nucleotides than about 6 kb, preferably fewer than about 1 kb which can be used as a probe. Such probes may be labeled with reporter molecules using nick translation, Klenow fill-in reaction, PCR or other methods well known in the art. After pretesting to optimize reaction conditions and to eliminate false positives, nucleic acid probes may be used in Southern, northern or in situ hybridizations to determine whether target DNA or RNA is present in a biological sample, cell type, tissue, organ or organism.

A "polypeptide" comprises a protein, oligopeptide or peptide fragments thereof.

A mutant, variant, or modified polypeptide means any polypeptide encoded by a nucleotide sequence that has been mutated through insertions, deletions, substitutions, or the like.

"Recombinant nucleotide variants" are alternate polynucleotides which encode a particular protein. They may be synthesized, for example, by making use of the "redundancy" in the genetic code. Various codon substitutions, such as the silent changes which produce specific restriction sites or codon usage-specific mutations, may be introduced to optimize cloning into a plasmid or viral vector or expression in a particular prokaryotic or eukaryotic host system, respectively.

"Linkers" are synthesized palindromic nucleotide sequences which create internal restriction endonuclease sites for ease of cloning the genetic material of choice into various vectors. "Polylinkers" are engineered to include multiple restriction enzyme sites and provide for the use of both those enzymes which leave 5' and 3' overhangs such as BamHI, EcoRI, PstI, KpnI and Hind III or which provide a blunt end such as EcoRV, SnaBI and StuI.

"Chimeric" molecules are polynucleotides or polypeptides which are created by combining one or more nucleotide peptide sequences (or their parts). In the case of nucleotide sequences, such combined sequences may be introduced into an appropriate vector and expressed to give rise to a chimeric polypeptide which may be expected to be different from the native molecule in one or more of the following characteristics: cellular location, distribution, ligand-binding affinities, interchain affinities, degradation/turnover rate, signaling, etc.

"Active" is that state which is capable of being useful or of carrying out some role. It specifically refers to those forms, fragments, or domains of an amino acid sequence which display the biologic and/or immunogenic activity characteristic of the naturally occurring peptide, polypeptide, or protein.

"Naturally occurring" refers to a polypeptide produced by cells which have not been genetically engineered or which have been genetically engineered to produce the same sequence as that naturally produced.

"Derivative" refers to those polypeptides which have been chemically modified by such techniques as ubiquitination, labeling, pegylation (derivatization with polyethylene glycol), and chemical insertion or substitution of amino acids such as ornithine which do not normally occur in proteins.

"Recombinant polypeptide variant" refers to any polypeptide which differs from naturally occurring peptide, polypeptide, or protein by amino acid insertions, deletions and/or substitutions.

Amino acid "substitutions" are defined as one for one amino acid replacements. They are conservative in nature when the substituted amino acid has similar structural and/or chemical properties. Examples of conservative replacements are substitution of a leucine with an isoleucine or valine, an aspartate with a glutamate, or a threonine with a serine.

Amino acid "insertions" or "deletions" are changes to or within an amino acid sequence. They typically fall in the range of about 1 to 5 amino acids. The variation allowed in a particular amino acid sequence may be experimentally determined by producing the peptide synthetically or by systematically making insertions, deletions, or substitutions of nucleotides in the sequence using recombinant DNA techniques.

An "oligopeptide" is a short stretch of amino acid residues and may be expressed from an oligonucleotide. Such sequences comprise a stretch of amino acid residues of at least about 5 amino acids and often about 17 or more amino acids, typically at least about 9 to 13 amino acids, and of sufficient length to display biologic and/or immunogenic activity.

A "standard" is a quantitative or qualitative measurement for comparison. Preferably, it is based on a statistically appropriate number of samples and is created to use as a basis of comparison when performing diagnostic assays, running clinical trials, or following patient treatment profiles. The samples of a particular standard may be normal or similarly abnormal.

An antibody or "specific binding parts" means any fragment of an antibody molecule that will bind antigen or other ligands such as lectins or other molecules. "Specific binding parts" is meant to include, but not be limited to antibody fragments such as Fab fragments, Fab'(2) fragments, Fc region fragments, Complimentarity determining regions (CDRs), Fv fragments, single chain Fv (scFv) fragments, and antigen binding site fragments.

A T cell "epitope" is an antigenic determinant recognized and bound by the T-cell receptor. Epitopes recognized by the T-cell receptor are often located in the inner, unexposed side of the antigen, and become accessible to the T-cell receptors after proteolytic processing of the antigen.

An "ELISpot Assay: Short for Enzyme-linked ImmunoSpot Assay", means an antibody based method to detect secretion of soluble factors released by cells. Originally developed as a method to detect antibody-secreting B-cells, later the method was adapted to determine T-cell reaction to a specific antigen, often represented as number of activated cells per million.

A Bovine MHC class II bioassay, means an assay measures IFN-γ release from T cells responding to specific stimulation through the ability of IFN-γ to induce and up-regulate expression of class II molecules on bovine endothelial cells. Bovine endothelial cells do not constitutively express class II molecules unless triggered by external signals such as IFN-γ.

Since the list of technical and scientific terms cannot be all encompassing, any undefined terms shall be construed to have the same meaning as is commonly understood by one of skill in the art to which this invention belongs. Furthermore, the singular forms "a", "an" and "the" include plural referents unless the context clearly dictates otherwise.

The invention is not to be limited only to the particular sequences, variants, formulations or methods described. The sequences, variants, formulations and methodologies may vary, and the terminology used herein is for the purpose of describing particular embodiments. The terminology and definitions are not intended to be limiting.
All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the above-described modes for carrying out the invention which are obvious to those skilled in the field of molecular biology or related fields are intended to be within the scope of the following outline of the invention ("claims").

The invention is further described in the following Annex and attached Appendix:

ANNEX A

CTL target antigen identification

1. **Materials and methods**

2. **Results**

1. Genome sequencing, preliminary annotation, selection of genes encoding candidate antigens

The Theileria parva nuclear genome consists of 4 chromosomes and was estimated to be 10 Mb in length.

The T. parva genome was sequenced using a whole genome shotgun strategy. T. parva genomic DNA (Muguga clone) was sheared and cloned into plasmid vectors. Both ends of randomly selected clones were sequenced. A total of 152,226 sequences with an average read length of 623 nt were obtained, equivalent to 9.48X coverages assuming a genome size of 10 Mb. Assembly of the sequences with "TIGR Assembler" produced a set of preliminary contigs represent 95% of the genome sequence.

Although our intention was to complete the T. parva genome sequence, the preliminary contigs were used to begin the process of identification of schizont antigens.

The preliminary contigs were loaded into an annotation database and subjected to an automated process that searched the T. parva contigs against a non-redundant database of proteins extracted from GenBank (called nraa). The search results were used to produce a set of T. parva gene models that encoded proteins similar to those in other organisms. This set of gene models was used to train the gene finding programs "GlimmerM" and "phat", which were subsequently run against all of the preliminary contigs to produce gene models for the entire preliminary genome sequence.

The proteins encoded by the predicted T. parva genes were subjected to a variety of analyses such as database searched ("blastp"), predictions of signal peptides and signal anchors ("SignalP 2.0"), and transmembrane domains ("TMHMM"). The results were reviewed and a set of ~55 genes encoding candidate antigens were selected for screening for immunogenicity. These gene sequences were screened and this led to the identification of antigens known as Tp2, Tp3, and Tp6.

2. Cloning of selected genes for screening for candidate antigens

1.1 RNA Extraction:

One million of *Theileria parva* (Muguga strain)-infected cells were washed with 1ml of Phosphate Buffered Saline (PBS) and were centrifuged at 15,000rpm for 10 seconds. After removing the supernatant, the cells were resuspended in 1ml of RNAzol® by pipetting. Then 100µl of chloroform were added and mixed briefly by vortex. The tube was spun at 15,000rpm for 15min at 4°C and 400µl of the upper aqueous phase were transferred to a new Eppendorf™ tube. 0.7 volume of Iso-propanol was added, vortex mixed and microfuged again at 15,000rpm for 10min at 4°C. After removing the supernatant, the pellet was washed with 300µl of 70% ethanol. After centrifugation at 15,000rpm for 5min at 4°C, 70% ethanol was aspirated and the pellet was resolved in 60µl of RNase free water. The concentration of the RNA was determined by a spectrophotometer.

1.2 One-step RT-PCR:

A Qiagen® One-step RT-PCR kit was used to amplify selected genes using RNA obtained above. Primers were designed to amplify the coding region of the selected schizont genes. Forward primers (FW!) are listed in Table 1 and reverse primers (RV1) are listed in Table2. The components mixed for a 50µl reaction for each set of primers. A master mix (5x RT-PCR buffer 10µl; dNTP mix 2µl; Enzyme mix 2µl; RNase inhibitor 1µl; RNA template 1µl; Water 32µl) was made less the primers and dispensed in labeled tubes, and then 1µl each of the corresponding FW1 and RV1 primers were added. The mixture was overlaid with mineral oil and placed in an MJ Research minicycler and the following program was applied.

Genes up to 1kb:

50°C 30 minutes; 95°C 15 minutes; 94°C 30 seconds; 55°C 30 seconds; 72°C 1 minute; 35 times from 94°C cycle and finally 72°C 10minutes

Genes between 1 and 2kb:

50°C 30 minutes; 95°C 15 minutes; 94°C 1 minute; 55°C 1 minute; 72°C 1 minute; 35 times from 94°C cycle and finally 72°C 10minutes

Genes between 2 and 3kb:

45°C 30 minutes; 95°C 15 minutes; 94°C 10 seconds; 55°C 1 minute; 68°C 3 minute; 35 times from 94°C cycle and finally 68°C 10minutes

1.3 Electrophoresis, Isolation and Purification of PCR products:

To the completed PCR reaction, 10µl of 6x loading dye was added and loaded onto 0.7% agarose gel containing 0.5µg/ml of Ethidium bromide. Electrophoresis was done at 150 volts for 30minutes. The DNA was visualized over an Ultra violet light illuminator and expected sizes of fragments were excised from the gel and

transferred to appropriately labeled tubes. Fragment DNA was extracted from gel by QIAquick Gel Extraction Kit from Qiagen. To the excised gel piece, buffer QG (three volume of the gel weight) was added and incubated at 50°C with intermittent shaking until dissolved. The dissolved gel was transferred to a QIAquick spin column in a provided 2ml collection tube, then 750µl at a time applied and spun for 1 minute to bind the column until all the gel solution were applied. The flow-through was discarded and the column replaced to the emptied collection tube. The column was washed with 750ul of wash buffer PE and spun for 1minute. The flow-through was discarded and spun again for 1 minute to dry. The column was transferred to a 1.5ml Eppendorf tube containing 5µl of 3M Sodium Acetate and then 50µl of buffer EB was pipetted into the column and left to stand for 1 minute before being spun for another minute. The eluted DNA was precipitated by addition of 150µl of ethanol, mixed well and incubated at –20°C for 20minutes. The tube was then spun in a microfuge at 15,000rpm for 15min at 4°C. The supernatant was aspirated then the residual pellet was washed with 300µl of 70% ethanol. Spun again in a cooled microfuge at 15,000rpm for 10min at 4°C. Aspirated again and ensured complete drying by being given another short spin and aspirated dry. The DNA was resuspended in 8µl of water and 1µl ran on a gel for quality and quantity assessment.

1.4 Ligation:

After determination the concentration of the fragments, an adjustment was made such that a ligation mix was set up at a molar ratio of 1:10 (vector:insert). [(pTargeT vector (Promega, 20ng/µl) 1µl; fragment 1-7µl (depend on the concentration of the fragments); 10x ligation buffer 1µl; T4 DNA ligase 1µl; water to make up final vol. to 10µl). After incubated at 4°C overnight, ligase was inactivated by incubation of the tube at 70°C for 30 minutes and then ethanol precipitated. Pellets were reconstituted in 10µl of water.

1.5 Transformation:

JM109 strain of *E. coli* electro-competent cells were used for transformation with the ligated samples. Preparation for electroporation was done by cooling the BIO-RAD® 0.2cm *E. coli* Pulser® Cuvette on ice, thawing the competent cells on ice, setting the electroporator equipment at 2.5KV, 200Ω and 25µF. Five µl of the ligation was added into 50µl of the competent cells, then quickly transferred into the cold cuvettes on ice, tapping firmly onto tissue paper to ensure all cells go to the bottom without leaving any spaces. The cuvette was wiped dry and pulsed till the PLS signal and an alarm signified successful pulsing. Pulsed cells were resuspended in 1ml of 2xYT medium immediately and then transferred into a Falcon 2059 tube and incubated for 1 hour at 37°C with shaking. When the incubation was over, the cells were plated on 2xYT plates containing 60µg/ml X-gal, 0.1mM IPTG, and 50µg/ml Ampicillin at a various dilutions. The plates were incubated at 37°C for overnight.

1.6 Colony screening by PCR:

V-bottom 96 microtiter plates were used for screening of the colonies. The PCR master mix was prepared such that each well has a total of 25µl of [1x PCR buffer mix 23.75µl; gene specific-forward primer 0.5µl; vector specific-reverse primer 0.5µl; Taq polymerase 0.25µl]. To screen the selected gene-containing plasmids, gene-specific primers (IF1, Table3) were used. The vector-specific reverse primer was 5'GAGCGGATAACATCACACAGG3'. Using a sterile tip, a colony was touched, then touched the master plate with the same tip at a place that is numbered (for identification purpose) and finally placed in the corresponding well, leaving it there until all the wells were completed. Using a pipette, the well contents were mixed then the tip was discarded and then followed with a drop of mineral oil in each well. The plate was placed in a MJ Research minicycler and run using the following cycles. 95°C 5 minutes; 94°C 20 seconds; 55°C 20 seconds; 72°C 30 seconds; Go To step two 34 more times; 72°C 5 minutes; 10°C infinite. At the end of the PCR, the plate was removed and 5µl of 6X loading dye was added to each well. The products were run on a 2% agarose gel. The positive clones on the master plates were identified and a sample taken for a 25ml overnight culture.

1.7 Midi-prep:

After the overnight growth of the 25ml culture, cells were spun down in a falcon tube at 3,000rpm for 10min at 4°C and processed as per QIAGEN® HiSpeed™ plasmid purification kit protocol. The pellet was resuspended in 4ml of buffer P1, added 4ml buffer P2, mixed well by inverting six times and left at room temperature for 5min. [During this time, QIAfilter cartridges were prepared - screwing the cap onto the outlet nozzle and placing in a convenient tube]. 4ml of chilled buffer P3 were added into the lysate and mixed immediately but gently by inverting 6 times. The neutralized lysate was poured into the barrel of the cartridge and incubated for 10min. Meanwhile, a HiSpeed midi tip was equilibrated by adding 4ml of buffer QBT and allowing the column to empty by gravity flow. The cap was removed from the outlet nozzle and the plunger gently inserted to filter through into the equilibrated HiSpeed tip. The cleared lysate was allowed to enter the resin by gravity flow then washed with 20ml buffer QC. DNA was eluted with 5ml buffer QF into a clean Falcon tube, followed by 3.5ml of 100% Iso-propanol and mixed well. This was then span at 3500rpm for 30minutes at 4°C, the supernatant discarded, the residual pellet resuspend the pellet in 1.5ml 70% ethanol and then transferred into a labeled 2ml Eppendorf tube. The tube was spun at 15000rpm for 10minutes at 4°C, aspirated and dried then reconstituted in 50µl water. A 1µl sample was run on an agarose gel for quantification and another 10µl were submitted for sequencing and *in vitro* assay (ELISpot and bioassay).

1.8 Sequencing:

Nucleotide sequence of pTargeT plasmids were determined by ABI PRISM® 3700 DNA Analyzer (Applied Biosystems) using vector-specific forward primer 5'ACGCCAGGATTTTCCCAGTCACG3' and vector-specific reverse primer 5'GAGCGGATAACAATTTCACACAGG3'.

Sequences of clones at various stages of cloning:

pTargeT5FW

CCCCAGTGCTGGGAGGTAATTGTAGTCATGAAGAACTAAAAAAATTGGGAATG
CTAGAGGGCGATGGTTTCGACAGGGATGCATTGTTCAAATCATCACATAGGTA
TGGGAAAGGGTAGGAAAAAGGTATGGTCTTAAAACTACTCCAAAAGTAGATA
AAGTCTTAGCAGATCTTGAAACACTGTTTGGAAAACACGGTCTTGGTGGTATT
AGTAAAGATTGTCTTAAATGTTTTGCACAAAGCCTAGTGTGCGTATTAATGAA
ATGTAGAGGAGCATGTCTCAAAGGACCATGTACTGACGACTGCCAAAATTGCT
TTGATAGAAACTGTAAATCTGCATTGCTGGAATGCATTGGGAAAACAAGTATT
CCAAATCCATGTAAATGGAAAGAAGATTATCTAAAATACAAATTTCCTGAAAC
AGATGAGGACGAATCTAC

pTargeT5RV

GCTTGGAATTCGCGGCCGCAGTCGACGGTACCCCCGGGGAAAGATTCTATGAA
GTGCCGGAGGCTTCTCCTTTTTTCGTAGATTCGTCCTCATCTGTTTCAGGAAATT
TGTATTTTAGATAATCTTCTTTCCATTTACATGGATTTGGAATACTTGTTTTCCC
AATGCATTCCAGCAATGCAGATTTACAGTTTCTATCAAAGCAATTTTGGCAGTC
GTCAGTACATGGTCCTTTGAGACATGCTCCTCTACATTTCATTAATACGCACAC
TAGGCTTTGTGCAAAACATTTAAGACAATCTTTACTAATACCACCAAGACCGT
GTTTTCCAAACAGTGTTTCAAGATCTGCTAAGACTTTATCTACTTTTGGAGTAG
TTTTAAGACCATACCTTTTTCCTACCTTTCCCATACCATGTGATGATTTGAACA
ATGCATCCCTGTCGAAACCATCGCCCTCTAGCATTCCCAATTTTTTTAGTTCTTC
ATGACTACCATTACCTCCCAGCACTGGGGAATGTAAAATGTAAATAATAAAGT
AAAGG

pTargeT12FW

CCTGTTTCTCACAGTTTTTAAAAAATGTGTCTGCTCTGAGGCGTAGTTCTCCAG
ATTTGTCACCAGATGGTTCTTTTCTTCAAGTAAAATCAGCTTCTCCTCAGGATA
AACAAGATGTAATCCAAAGTTCCTCTCCTAAGGTAACAGTGCCTACGGTTGAC
CCTGAAGGCCTCAAGAAGGCGGTTACTGCAGCAGTTCTATCAAACCAAAATCA
AGCTCTACAAAACGGTGCTCTTAATCCAGCAGATTTCACTCAAGCTGCCTCTGT
TAATTCCATGAGTAATGCTGTTAGTGCCATGAACAATACTGTTGGTCCAGTAA
AAAATCCCATGGCTACTGTTGGTACTATGAACTCCTTTACTGGAATGCCTGGTG
TACAGGATAATTTTCCTCAGACACCGCCTGTTAATGTTCAAGACACCTCTACCC
AAGAGAACAGTCTTGACAACCTAAATCTCCTCTTAGATCCCTCGTTAGTAAAG
ATATCTCAAGCTGATAGTCACATAAAAGAAAGCATGGAAAAAGCTGTACACA
GCCTTAAAAAGGTCTTGGAGGGGCTAACCAACCTTGCGACTCTGTCTAAAAGT

AGGGATACTGAACCGTTTAATGTTCTGGGGGATGACTATACGATGCGTAACGT
TTTGGACCTCATGAATAAGGAACTCAGG

pTargeT12RV

GGCCGCAGTCGACGGTACCCCCGGGGAAAGACTTTAGGATTTTTTATTATCGT
CTGGACTCTTAGTGAAGGTGGAAAGTGCAAAGGCGTTGAATTGGAACACAACT
TTCTGAAGAGATTCAACCTGCCTGAGTTCCTTATTCATGAGGTCCAAAACGTTA
CGCATCGTATAGTCATCCCCCAGAACATTAAACGGTTCAGTATCCCTACTTTTA
GACAGAGTCGCAAGGTTGGTTAGCCCCTCCAAGACCTTTTTAAGGCTGTGTAC
AGCTTTTTCCATGCTTTCTTTTATGTGACTATCAGCTTGAGATATCTTTACTAAC
GAGGGATCTAAGAGGAGATTTAGGTTGTCAAGACTGTTCTTCTTGGGTAGAGG
TGTCTTGAACATTAACAGGCGGTGTCTGAGGAAAATTATCCTGTACACCAGGC
ATTCCAGTAAAGGAGTTCATAGTACCAACAGTAGCCATGGGATTTTTTACTGG
ACCAACAGTATTGTTCATGGCACTAACAGCATTACTCATGGAATTAACAGAGG
CAGCTTGAGTGAAATCTGCTGGATTAAGAGCACCGTTTTGTAGAGCTTGATTTT
GGTTTGATAGAACTGCTGCAGTAACCGCCTTCTTGAGGCCTTCAGGGTCAACC
GTAGGCACTGTTACCTTTAGAGAGGAACTTTG

pTargeT19FW

GTGATTGCCGCCACCATGGCTCAGATTCCTGTTGATAAATTCGCTAAATTAGTT
ACTGGAGCCGGCTCTGCTCTTTTATTATTCGGTTCAGGTGCCTGGCTTGTCAAT
TCCAGTTTATACGATGTTGGAGCTGGGCATAGAGCTGTTGTATATAACCGTATC
ACTGGAATAAGTGAGACTACACATGGAGAAGGAACGCACTTCATAATTCCCTG
GCTAGAACGTCCAATAATTTACGATGTGAGGACTCGTCCTAGGACTCTGATGT
CTCTCACCGGAAGCCGTGACTTGCAGATGGTTAACATCACCTGCCGTGTGTTGT
CTCGTCCCGATGAGCGCAGACTCAGGGATATTTACAGGCACTTGGGCAAAGAT
TACGACGAGCGAGTCCTGCCTTCAATAATAAACGAGGTTCTGAAGAGTATTGT
GGCCCAGTACAACGCCTCTCAGCTCATTACTCAGAGAGAAAGAGTTAGCAAAG
CAGTCAGGGACCAGCTGGTGAACAGGGCCAGGGACTTTAATATTCTTCTCGAT
GATGTCTCCTTAACCCACTTAAGCTTCAGTCCTGAATATGAAAAGGCTGTAGA
GGCTAAACCAGTAGCTCAACAGCAAGCTGAACGCAGTAAATATATAGTGTTGA
AGGCTCAGGAG

pTargeT19RV

GCTTGGAATTCGCGTCCGCAGTCGACGGTACCCCCGGGGAAAGATTTTATTTA
TCAGTTGAGAGTAAGAGAGTATTACTATTGAGCATGATTTTATTCTGCGATTTG
GAGAGAATGTTAGCCACTTCCTTAGCGGTTTCAATTCTCCGAAGCGTAATAAA
GGCAGGGTTATCCTTAATTGCACTTCCAATAAGCCTTGCAGCCTCAGACTCTCC
CTGAGCCTTAATTATCGTCGATTTCTTCTCCTCCTGAGCCTTCAACACTATATAT
TTACTGCGTTCAGCTTGCTGTTGAGCTACTTGTTTAGCCTCTACAGCCTTTTCAT
ATTCAGGACTGAAGCTTAAGTGGGTTAAGGAGACATCATCGAGAAGAATATTA
AAGTCCCTGGCCCTGTTCACCAGCTGGTCCCTGACTGCTTTGCTAACTCTTTCT
CTCTGAGTAATGAGCTGAGAGGCGTTGTACTGGGCCACAATACTCTTCAGAAC

CTCGTTTATTATTGAAGGCAGGACTCGCTCGTCGTAATCTTTGCCCAAGTGCCT
G

Table 1 (Forward primers)

Gene No.	Name	Sequence (5' to 3')
2	299FW1	GCCGCCACCATGAAATTGGCCGCCAGATTAATTAGCC
3	254FW1	GCCGCCACCATGAAATTAAATACTATCGCAATAGCCTTT
6	312FW1	GCCGCCACCATGGCTCAGATTCCTGTTGATAAATTCG

Table 2 (Reverse primers)

Gene No.	Name	Sequence (5' to 3')
2	299RV1	CTATGAAGTGCCGGAGGCTTCTCC
3	254RV1	TTAGGATTTTTTATTATCGTCTGGACTC
6	312RV1	TTATTTATCAGTTGAGAGTAAGAGAGTATTA

Table 3 (Internal forward primers)

Gene No.	Name	Sequence (5' to 3')
2	299IF1	TCCATGTAAATGGAAAGAAGATTATC
3	254IF1	GGAACTCAGGCAGGTTGAATCTCTTC
6	312IF1	CCGCTAAGGAAGTGGCTAACATTC

3. Materials and methods related to screening for candidate antigens

3.1 Immunisation & details of cattle
3.2 Generation, characterization and maintenance of CTL.

- Establishment of *T. parva* infected cell lines
- Generation of *T. parva* bulk cultures
- Testing and characterizing cytotoxic activity
- Generation of *T. parva* specific $CD8^+$ CTL polyclonal lines and clones
- Maintenance and expansion of TpM specific CD8+ CTL polyclonal lines and clones.

3.3 Establishment and maintenance of bovine skin fibroblasts
3.4 Establishment and maintenance of bovine testicular endothelial cells
3.5 Immortalization of bovine skin fibroblasts and endothelial cells
3.6 Transfection of COS-7 cells and immortalized skin fibroblasts with schizont cDNA pools and selected genes
3.7 Detection of CTL recognition of transfected SF and COS cells by IFN-γ ELISpot
3.8 Detection of CTL recognition of transfected SF and COS cells by IFN-γ bioassay
- Standard EC bioassay
- Generation of test supernatants for bioassay from specific activation of *T. parva* –specific $CD8^+$, cytolytic T cell lines
- Indirect fluorescent antibody assay for EC class II MHC expression

3.9 Detection of CTL lysis of transfected iSF and COS-7 cells.
3.10 Identification of Tp2 CTL epitopes with synthetic peptide libraries
3.11 Detection of Tp2 specific *ex vivo* $CD8^+$ T cell responses from immune cattle after challenge with *T. parva*

4. Results

4.1 Optimization of INF-γ ELISpot for the recognition of target antigens by schizont specific CTL.
4.2 Identification of Tp2 following the screening of selected genes with BW002, BW013 and BW014 CD8+ polyclonal lines and D409 CTL clone #10
- Mapping of the Tp2 CTL epitopes.
- Kinetics of Tp2 specific $CD8^+$ T cell responses from immune cattle following challenge with *T. parva* sporozoites.

4.7 Expression of the candidate antigens.

Immunisation & details of cattle

The 10 cattle used in this invention were pure bred *Bos indicus* (Boran) pure bred *Bos taurus* (Jersey and Friesian) or crossbreeds. Details of the animals are shown in Table 4. Cattle were immunized by 'infection and treatment' against the Muguga stock of *T. parva* by simultaneous inoculation of sporozoites and long-acting oxytetracycline at 20mg/kg BW (Radley et al 1975). Cryopreserved sporozoites (Stabilate # 4133) were thawed and diluted 1/20 as previously described. Animals were given a subcutaneous injection of 1ml of diluted sporozoites 2cm above the right parotid lymph node. Animals were monitored daily for changes in rectal temperature and from day 5 post challenge lymph node biopsies were taken using a 21G needle. Giemsa stained biopsy smears were examined for the presence of schizont infected cells and scored on a scale of 1-3. Animals suffering from moderate reactions were treated with Buparvaquone (Butalex, Mallinckrodt Veterinary Ltd, UK).

Animal #	Breed	Date of birth	Sire	Dam
BV050	Boran	9/12/99	291	2772
BV057	Boran/Jersey	3/7/00	NK	AT230
BV115	Friesian	NK	NK	NK
BW002	Jersey	NK	NK	NK
BW012	Boran/ Friesian	18/8/00	Mystig	1507
BW013	Guernsey/ Ayrshire	8/9/00	D14	D646
BW014	Guernsey/ Ayrshire	15/9/00	D14	D638
BX063	Boran	1/4/01		BN59
BX065	Boran	3/4/01	282	BN59
D409	Guernsey	6/12/85	1422	D23
F100	Boran	17/3/88	103	1130

Table 4. Details of animals used in study. Animals were screened for *T. parva* specific antibody and only sero-negative animals recruited. Animals were immunized against *T. parva* (Muguga) by a live infection and treatment regime. NK = Not known.

Generation, characterization and maintenance of CTL.

ESTABLISHMENT OF *T. PARVA* INFECTED CELL LINES

Prior to immunization, venous blood was collected from the eleven animals, PBMC were purified and infected *in vitro* with *T. parva* (Muguga) sporozoites (Godeeris & Morrison, 1986). Infected cell lines were maintained in TpM medium (RPMI-1640 supplemented with 10% Fetaclone II (Hyclone, UK; tested for BVDV & *Mycoplamsa spp.*), 100iU/ml Penicillin, 100µg/ml Streptomycin, 50µg/ml Gentamycin, $5x10^{-5}$M 2-mercaptoethanol and 2mM L-Glutamine) and passaged 1/5 three times a week.

GENERATION OF *T. PARVA* SPECIFIC BULK CULTURES

Venous blood from immunized animals was collected from 4 weeks post-immunization. PBMC were prepared as described previously (Goddeeris & Morrison, 1988). PBMC were adjusted to $4x10^6$/ml in CTL medium (RPMI-1640 without HEPES supplemented with 10% FBS (HyClone; tested for BVDV & mycoplamsa spp.), L-glutamine, 2-mercaptoethanol and antibiotics as described above) and 1ml/well added to 24 well plates (Costar, Corning, NY, USA). PBMC were co-cultured with irradiated (50Gy) autologous *T. parva* infected cells (TpM) at 2×10^5 /well and incubated for 7 days at 37°C in a 5% CO_2 humidified atmosphere. Cells were harvested by aspiration and dead cells removed by centrifugation over Ficoll-Paque Plus (Amersham Pharmacia Biotech, Uppsala, Sweden). After washing in CTL medium, cells were added to 24 well plates ($3x10^6$/well) and co-cultured with irradiated PBMC (filler cells) at 1 $x10^6$/well and TpM at 2 $x10^5$/well for 7 days as before. Viable cells were harvested as described above, adjusted to 2 $x10^6$/well and stimulated with 4 $x10^5$/well irradiated TpM and 2 $x10^6$/well irradiated autologous PBMC as filler cells.

TESTING AND CHARACTERIZING CYTOTOXIC ACTIVITY

^{51}Chromium Release Assay: Autologous and allogeneic TpM in log phase of growth were resuspended at $2x10^7$/ml cytotoxicity medium (RPMI-1640 medium with 5% Fetaclone II). 100µl of the target cells were mixed with 100µl (100µCi) of ^{51}Cr-sodium chromate and incubated for 1 hour at 37°C. Cells were washed 3 times in 7ml of cytotoxicity medium by centrifugation at 1500 rpm for 7 min at RT and resuspended at $1X10^6$/ml. Viable cells were harvested from TpM stimulated lines 7 days post-stimulation (effector cells) and resuspended in cytotoxicity medium at $2x10^7$/ml. Two-fold doubling dilutions of effector cells were distributed in duplicate (100µl/well) to 96-well half area (A/2) flat-bottom culture plates (Costar, Corning, NY, USA) resulting in a range of effector cell concentrations of $4x10^6$ to $2.5x10^5$/well. Target cells were added to each well containing effector

cells (50µl/well) resulting in target cell ratios ranging from 80:1 to 5:1. In separate triplicate wells target cells were added to 100µl cytotoxicity medium or 1% Tween20 to measure spontaneous and maximal release of the label respectively. Plates were incubated for 4hours at 37°C in 5% CO_2 humidified atmosphere. Cells were resuspended in wells by repeated pipetting and pelleted by centrifugation at 180X*g* at room temperature. 75µl of supernatant was transferred from each well into sample vials (Milian, Geneva, Switzerland) and gamma emissions counted in a gamma counter (Micromedic MEplus, TiterTek, Huntsville, AL, USA). Results were calculated and expressed as percent cytotoxicity (= 100 x (test release – spontaneous release) / (maximum release – spontaneous release).

Evidence for MHC class I restricted lysis was assessed by the capacity of monoclonal antibodies recognizing bovine MHC class I to inhibit lysis. Cells were prepared for the cytotoxicity assay as described above except that target cells were resuspended at double the density ($2x10^6$/ml) and 25µl added first to the plate. 25µl of either cytotoxicity medium or monoclonal antibody (mAb; IL-A88 diluted 1/15 in cytotoxicity medium was added to target cells and incubated for 30min at room temperature. Serial dilutions of effector cells were then added as described above.

GENERATION OF *T. PARVA* SPECIFIC CD8+ CTL POLYCLONAL LINES AND CLONES.

Viable cells from TpM stimulated cultures were harvested day 7 post-infection and $CD8^+$ T cells were isolated either by positive selection using flow cytometry (FACStar Plus, BD Biosciences, San Jose, CA, USA) or negative selection using Dynabeads (Dynal Biotech, Bromborough, UK).

Positive selection: Cells were adjusted to $2x10^7$/ml in sterile monoclonal antibody IL-A105 (specific for bovine CD8) diluted 1/100 and incubated for 30 minutes at 4°C. Cells were washed twice in cold culture medium and resuspended at $2x10^7$/ml in sterile goat anti-mouse polyvalent immunoglobulins conjugated to FITC (Sigma). Cells were washed twice in cold medium before being run through a FACStar plus cell-sorter and $CD8^+FITC^+$ cells collected.

Negative selection: Cells were adjusted to $2x10^7$/ml in sterile mAbs IL-A12 (specific for bovine CD4) and GB21A (specific for bovine γδ TCR) diluted 1/100 and incubated for 30 minutes at 4°C. Cells were washed twice in cold PBS and resuspended $1.4x10^7$/ml in sterile PBS containing washed sheep anti-mouse IgG Dynabeads according to the manufacturers instructions Cells and beads were rotated for 30 min at 4°C and Dynabead rosetted cells were collected by placing the sample tube in a Dynal Magnetic Particle Concentrator (MPC) for 5 min, the supernatant was removed, transferred to another sample tube and residual Dynabead rosetted cells removed by another incubation in a Dynal MPC. The supernatant was removed and cells washed twice in complete medium.

Cloning: CD8+ T cells enriched by either of the methods described above were adjusted to 30, 10, and 3 cell/ml and distributed into 96-well, round bottom culture plates containing 2 x 10^4 irradiated autologous TpM, 5 x 10^3 irradiated autologous PBMC and 5U/ml recombinant human IL-2 (HuIL-2; Sigma, Poole, UK) in a final volume of 200µl/well. Plates were incubated at 37°C in humidified incubator containing 5% CO_2 in air. Wells showing significant cell growth were selected for analysis of lysis of autologous TpM in an (^{111}In) Indium oxine release assay. The ^{111}In release assay was performed as described above for ^{51}Cr release assay except that targets cells were labeled by addition of 5µCi ^{111}In/1 x 10^6 cells and incubation for 15 minutes at 37°C. Cells were washed five times with cytotoxicity medium and resuspended at 1x10^5/ml and added 50µl/well to 96-well V-bottom 96 well plates. 100µl cells from wells showing significant growth were transferred to 96-well V-bottom culture plates (Greiner) and centrifuged (180x*g*) for 5 min. Cells were resuspended 100µl/well in cytotoxicity medium and transferred to wells containing labeled target cells. Plates were centrifuged as described above to pellet cells before being incubated for 4 hours at 37°C in 5% CO_2 humidified atmosphere.

Maintenance and expansion of TpM specific $CD8^+$ CTL polyclonal lines and clones.

CTL populations that exhibited lytic activity on autologous infected cells and originated from cell dilutions that gave rise to cell growth in less than 30% of the wells, as they have high probability of being clones, were selected for expansion. The remaining cells were harvested from each well and resuspend them in culture medium (without HEPES) at a concentration estimated to be between 500 and 5000 cells/ml. 100µl of cell suspension was distributed into 96 well, round bottom culture plates. 100µl of autologous irradiated TpM at 5 x10^4 autologous irradiated TpM in medium containing 5U/ml HuIL-2. Between day 14 and 21 post-stimulation clones and polyclonal CTL lines were subcultured in 96 well, round bottom culture plates by co-culturing 5000 CTL/well with 25,000 autologous irradiated TpM and 5U/ml HuIL-2 in a final volume of 200µl/well.

ESTABLISHMENT AND MAINTENANCE OF BOVINE SKIN FIBROBLASTS

A skin biopsy was taken aseptically from the ears of cattle and placed in a 50ml falcon tube containing Alsever's solution. In the laboratory laminar flow hood, the biopsy was placed into sterile Petri dishes (Sterilin) containing 1ml of 0.25% Trypsin-EDTA. Using a sterile scalpel blade, the sample was cut into small pieces and placed into a 50 ml falcon tube containing Trypsin-EDTA. The preparation was placed in a shaking incubator for 1-1.5 hr at 37°C with gentle continuous shaking. This facilitates detachment of cells. This digestion was stopped by addition of 1ml heat-inactivated FBS. The cell suspension was centrifuged for 10 min at 200×*g*

and the cell pellet re-suspended in 6 ml of Dulbecco's minimum essential medium, DMEM (Gibco-BRL, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT), 400 IU/ml penicillin, 300 μg/ml streptomycin and 2 mM L-glutamine. This cell suspension was seeded in 5ml amounts into 25-cm^2 flasks and incubated at 37°C in a 5% CO_2 humidified atmosphere. Cultures were examined microscopically after every 4 days for growth and half the medium was replaced with fresh one until growing colonies were evident. Once positive colonies were identified they were rinsed with Ca^{2+}- and Mg^{2+}-free PBS/EDTA (0.02% EDTA) and detached by 2 min incubation at 37°C with Trypsin-EDTA solution containing 2.5 mg/ml Trypsin and 0.2 mg/ml EDTA in HBSS (Sigma). Following a wash in complete DMEM containing 10% FBS the skin fibroblasts (SF) were passaged into 25-cm^2 tissue culture flasks (Costar). Cells were maintained in complete DMEM containing 10% FBS, 200 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine and passaged every 3 days at a ratio of 1:3. Cells were expanded in 75-cm^2 tissue culture flasks (Costar) until confluent yielding 3-4×10^6 cells/flask. For consistency a liquid nitrogen cell bank was prepared with 2×10^6 cells/freezing vial in 10% DMSO in FBS.

ESTABLISHMENT AND MAINTENANCE OF BOVINE TESTICULAR ENDOTHELIAL CELLS

Bovine testicular vein or pulmonary artery EC lines were established as described by Byrom and Yunker (1990) with the modifications of Mwangi et al (1998). Briefly, the vein was placed in wash buffer consisting of Ca^{2+}- and Mg^{2+}-free PBS supplemented with 400 IU/ml penicillin, 400 μg/ml streptomycin and 5μg/ml fungizone. The vessel was then slit longitudinally, washed twice before being cut into 1-cm^2 pieces and then placed lumen side down on a drop of collagenase (1 mg/ml) and incubated for 1 h at 37°C. The cell suspension was centrifuged for 5 min at 200×*g* and re-suspended in 24 ml 2× complete DMEM (Gibco-BRL, Paisley, UK) supplemented with 20% heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT), 400 IU/ml penicillin, 300 μg/ml streptomycin, 5 mg/ml fungizone, 300 μg/ml endothelial growth supplement (Sigma, St Louis, MO) and 2 mM L-glutamine. One ml of the cell suspension was seeded in each well of a 24-well tissue culture plate (Costar, Cambridge, MA, USA). Once confluent, monolayers in each well were rinsed with Ca^{2+}- and Mg^{2+}-free PBS/EDTA (0.02% EDTA) and detached by 2 min incubation at 37°C with 0.25% trypsin/EDTA solution containing 2.5mg/ml trypsin and 0.2 mg/ml EDTA in HBSS (Sigma). Following a wash in DMEM containing 10% FBS the cells were passaged into 25-cm^2 tissue culture flasks (Costar). Cells were maintained in complete DMEM containing 10% FBS, 200 IU/ml penicillin, 100 μg/ml streptomycin, 2.5 mg/ml fungizone, 2 mM L-glutamine and passaged every 3 days at a ratio of 1:3. Cells were expanded in 75-cm^2 tissue culture flasks (Costar) until confluent yielding between 3×10^6 and 4×10^6 cells per flask. For consistency a liquid nitrogen cell

bank was prepared with 2×10^6 cells per freezing vial. Cells were raised from nitrogen into 75-cm^2 flasks and used between the fourth and tenth passages.

IMMORTALIZATION OF BOVINE SKIN FIBROBLASTS AND ENDOTHELIAL CELLS

Cells were immortalized with the SV40 early region gene by transfection of an expression plasmid psvNeo (ATCC code 37150). The product is supplied as a freeze dried to which 300ul of 2XYT with ampicillin was added and fully resuspended. The 300ul suspension was sub-cultured into 6mls and divided into 3mls each. The cultures were incubated overnight at 37°C and then subcultured again into 50mls of 2XYT with ampicillin overnight at 37°C. Maxiprep of psvNeo plasmid were made for use in immortalizations. Plasmid DNA for transfection was standardized to 2mg/ml.

The DNA mix was prepared by mixing 5µl DNA with 495µl of DMEM with antibiotics but no serum. A working dilution of Fugene 6 transfection reagent (Roche) was prepared by mixing 15µl of Fugene with 485µl of DMEM. Mixing was done using the 2ml sterile non-pyrogenic, DNAse and RNAse free cryopreservation tubes. The diluted Fugene was added to the diluted DNA drop wise with constant tapping at the end of the tube. The Fugene-DNA complex was allowed to form at room temperature for 30 min.

Cells were cultured in 6-well plates (Costar) at a density of 2×10^5 cells per well and grown to confluence overnight at 37°C, 5% CO_2 in a humidified incubator. Culture medium was removed completely from the monolayers to be transfected. The Fugene-DNA complex was then added gently onto the monolayer. Plates were incubated at 37°C, 5% CO_2 in a humidified atmosphere for 3-4 hours. The transfection complex was removed; fresh DMEM medium containing 10% fetal calf serum was added and then cultured for a further 72h. Cells from each well were rinsed with PBS/EDTA, detached with Trypsin/EDTA, washed and re-suspended in complete DMEM and sub-cultured into one T-25 flask (Costar). After incubation for 2-3h, normal DMEM medium was removed and replaced with a selection DMEM medium containing 10% FCS and 0.5µg/ml of G418 (2.5µg/ml G418 for endothelial cells) and incubated further. Upon observation of high death rate of cells, half the medium was replaced with fresh selection medium until growing colonies were evident. This process took 3-4 weeks depending on the cell lines. Positive colonies were the sub-cultured into 24 well plates and then to T-25 flasks. Immortalization was confirmed by checking for expression of large T-antigen using an anti-SV40 antibody conjugated to HRP. Further expansion and maintenance of the cells was carried out using complete DMEM.

TRANSFECTION OF COS-7 CELLS AND IMMORTALIZED SKIN FIBROBLASTS WITH SCHIZONT cDNA LIBRARY AND SELECTED GENES

COS-7 cells and iSF were maintained in T75 and T150 TC flasks with DMEM supplemented with 10% FCS, 2mM L-glutamine and antibiotics (TC medium) as described above. Cells are split 1:4 every 3 days. The day prior to transfection, cells were harvested by the removal of medium, washing in PBS and incubation in 0.25% Trypsin-EDTA for 5 min at 37°C. Once cells had detached TC medium was added and cells removed. Cells were washed by centrifugation at 1200 rpm for 10min and resuspended in TC medium. A viable cell count taken, density adjusted to $2.0x10^5$/ml, cells dispensed, 100µl/well, into 96 well flat-bottom TC plates and incubated for overnight at 37°C in a CO_2 (5%) humidified incubator.

DNA was prepared for either single transfections of SF or double transfections (co-transfection of schizont cDNA and BoLA class I cDNA) of COS cells. 6µl of schizont cDNA and 6µl of BoLA class I cDNA (for co-transfection) at 50ng/µl in dH_2O were added to 150µl unsupplemented DMEM in wells of a 96-well round-bottom plate. FuGENE 6 transfection reagent was pre-warmed to 37°C. 0.9µl or 0.45µl FuGENE 6 was added to each well for double and single transfections respectively. The well contents mixed by shaking on a Dynatech Varishaker for 1 min and incubated at RT for 20 min. The medium from the 96 well plates containing adherent COS cells and SF was removed and each transfection complex added to triplicate wells (50µl/well). The cells were then incubated for 4 hours at 37°C in a CO_2 (5%) humidified incubator. The transfection complex was removed and replaced with 200µl/well DMEM supplemented as described above and incubate for 24 or 48 hours at 37°C in a CO_2 (5%) humidified incubator.

DETECTION OF CTL RECOGNITION OF TRANSFECTED SF AND COS-7 CELLS BY IFN-γELISPOT

24 hours post-transfection, medium was removed from wells containing transfected cells, cells were washed cells with PBS (200µl/well) and detached by the addition of 100µl/well Trypsin-EDTA as described above. Once the cells had detached the contents of each well were transferred to a 96 well round-bottom plates containing 100µl/well cold RPMI with no HEPES supplemented with 10% FCS (CTL medium). The cells were centrifuged at 1200 rpm for 3 min; supernatant removed and resuspended 50µl/well in CTL medium

Schizont specific CTL, generated and maintained as described above, were harvested 7-14 days post-stimulation, transferred to polycarbonate tubes, pelleted at 1200 rpm for 10min and resuspended at $2x10^5$/ml in CTL medium supplemented with 5U/ml HuIL-2 (Sigma).

ELISpot plates (Millipore corporation, Bedford, MA, USA) were coated 50µl/well with 2µg/ml of murine anti-bovine IFN-γ mAb (CC302; Serotec, UK) and incubated overnight at 4°C. Wells were washed twice with unsupplemented RPMI-1640 and blocked 200µl/well with RPMI-1640 supplemented with 10% FBS by incubating at 37°C for 2 hours. The blocking medium was removed and replaced with 50µl/well CTL and 100µl/well transfected cells. As a positive control, irradiated TpM are serially diluted in COS cells or SF with each at a density of $4x10^5$/ml. and

populations containing 32, 16, 8, 4, 2, 1% TpM are added 50µl/well to wells containing CTL. Plates were incubated in a humidified incubator at 37°C for 20 hours. After incubation, the contents of wells were removed and wells washed four times with sterile distilled water supplemented with 0.05% Tween 20 per well and the plate shaken on a shaker for 30 seconds between washes. The process was repeated an additional four times, using PBS supplemented with Tween 20 (PBS-T). Wells were then incubated with 100µl/well rabbit anti-bovine IFN-γ antisera diluted 1/1500 in PBS-T supplemented with 0.2% BSA (PBS/BSA) for 1 hour at room temperature. Wells were washed 4 times with PBS-T before being incubated for 1 hour at room temperature with 100µl/well murine monoclonal anti-rabbit IgG conjugated to alkaline phosphatase (Sigma) diluted 1/2000 in PBS-T/BSA. Sigma Fast BCIP/NBT buffered substrate (Sigma) was by dissolving 1 tablet/10ml dH_2O and passing it through a 0.2µm filter. Plates were washed six times as described above with PBS-T, 100µl/well BCIP/NBT substrate added and plates incubated for 10minutes at room temperature in the dark. The substrate was then removed, wells washed with copious amounts of H_2O and plates air-dried at room temperature in the dark. Plates were finally read on an automated ELISpot reader (AID Diagnostica, Strasberg, Germany).

BIOASSAY FOR CTL ACTIVATION BASED ON RAPID INDUCTION OF CLASS II MHC EXPRESSION BY CONSTITUTIVELY NEGATIVE BOVINE ENDOTHELIAL CELLS

Standard EC bioassay

Endothelial cells were detached from confluent 75-cm^2 tissue culture flasks by treatment with trypsin/EDTA as described. 2.5×10^4 cells were seeded into each well of a 48-well plate in 1 ml of complete DMEM and incubated overnight. Cell-free test supernatants derived from either co-culture of *T. parva*-specific $CD8^+$ T cell lines and SF transfected with test genes or recombinant bovine IFN-γ (rBoIFN-γ, the kind gift of Ciba-Geigy, Basel, Switzerland) were dispensed in duplicate wells in a final volume of 160 µl per well.

Generation of test supernatants for bioassay from specific activation of *T. parva* –specific $CD8^+$, cytolytic T cell lines

T. parva-specific $CD8^+$ CTL lines were generated and maintained using methods initially described. Test supernatants were collected from 96-well flat-bottomed microtitre plates (Costar) 48 hours after restimulation of resting T cell lines (2×10^4 per well) with 4×10^5 COS-7 cells co-transfected with the KN104 gene and test gene(s) or confluent autologous SF transfected with the test gene(s) in a final volume of 200 µl for 24 h. These tests were set-up at least in duplicates. Where indicated, class I MHC was blocked using a specific antibody (IL-A88) to check for MHC class I restriction of the CTL lines. Additional negative control supernatants were derived from co-culture of CTL with untransfected immortalized SF or COS-7

cells. Positive control supernatants were obtained from co-culture of CTL with varying proportions of irradiated autologous TpM.

Detection of CTL lysis of transfected iSF and COS-7 cells.

Autologous iSF or COS-7 cells were seeded in 6-well plates (Costar) at a density of $2.5x10^5$ /well and incubated for 2 hours at 37°C to allow cells to adhere. For single transfections of iSF, 2μg of test or control cDNA was added to 1ml unsupplemented DMEM containing 3μl FuGENE 6 transfection reagent and incubated for 40min. For COS-7 cells, 2μg of test or control cDNA was added with 2μg of BoLA class I cDNA to 1ml DMEM containing 6μl FuGENE. 1ml of DNA/Fugene complex was added per well of adherent COS-7 or iSF. Plates were incubated for 4 hours at 37°C, the transfection complex was removed and replaced with 2ml/well of complete DMEM and the plates incubated for a further 20 hours at 37°C. Transfected cells were harvested by removal of medium, washing in PBS, detachment by Trypsin-EDTA and washing in complete DMEM. Transfected cells and TpM were labeled with ^{51}Chromium and the ability of schizont-specific CTL lines (day 6-8 post-stimulation) to lyse these targets was assessed as described above.

IDENTIFICATION OF Tp2 CTL EPITOPES WITH SYNTHETIC PEPTIDE LIBRARIES

Peptide libraries (Cleaved PepSets; Mimotopes, Clayton, Australia) were generated for the Tp2- HD6 restricted CTL epitope. The PepSet libraries contained every 12mer, 11mer, 10mer and 9mer offset by 2 amino acids from the protein sequences. However, the peptides were prepared by truncations of the 12mers at the N-terminus and were supplied lyophilized with each tube containing a nominal 12mer and the 9, 10, 11mer truncations with the same C-terminus. Peptides were dissolved in 400μl 50% (v/v) DNA synthesis grade acetonitrile/water (Applied Biosystems, Warrington, UK). To aid the dissolution, tubes were held in a sonicator water bath for 2 x 10 min. Peptides were aliquoted into labeled cryopreservation tubes (Greiner) and stored at –20°C. For screening with CTL, peptides were prepared at 10μg/ml in complete RPMI-1640 and 10μl added to triplicate wells of an ELISpot plate, coated, washed and blocked as described above. Autologous iSF or P815 cells stably expressing the BoLA class I HD6 (P815-HD6) or JSP-1 (P815-JSP-1) were adjusted to a density of $4x10^5$/ml and 50μl added to wells containing peptides. The plates were incubated at 37°C for 1 hour before CTL, prepared as described above for screening transfectants, were added 50μl/well. Plates were incubated for 20 hours at 37°C and then developed as described above. Based on the results of the screening with the Tp2 PepSets, individual 9, 10 and 11mer peptides were synthesized in order to define the CTL epitopes. Peptides were prepared and screened using the IFN-γ ELISpot as described above.

Peptide-pulsed iSF and BoLA class I P815 transfectants were prepared as targets for ^{51}Chromium release assays by incubating $2x10^6$ iSF or P815 cells overnight in

T25 tissue culture flasks (Costar) with Tp2 peptides diluted to 1μg/ml in complete DMEM. Cells were harvested, labeled and assayed as described above.

DETECTION OF Tp2 SPECIFIC *EX VIVO* $CD8^+$ T CELL RESPONSES FROM IMMUNE CATTLE AFTER CHALLENGE WITH *T. PARVA* SPOROZOITES.

Cattle, BW002, BW013 and BW014, whose schizont specific CTL lines had been shown to recognize Tp2, were challenged with a lethal dose of *T. parva* (Muguga) sporozoites. Cryopreserved sporozoites (Stabilate # 4133) were thawed and diluted 1/20 as previously described. Animals were challenged by subcutaneous injection of 1ml of diluted sporozoites 2cm above the right parotid lymph node. Animals were monitored daily for changes in rectal temperature and from day 5 post challenge lymph node biopsies were taken using a 21G needle. Giemsa stained biopsy smears were examined for the presence of schizont infected cells and scored on a scale of 1-3. Animals were bled on day 2 and daily from day 6 to 13 and PBMC were isolated as described above. $CD8^+$ T cells and $CD14^+$ monocytes were purified from PBMC by MACS magnetic cell sorting according to the manufacturers instructions (Miltenyi Biotec, Gergisch Gladbach, Germany). $CD8^+$ T cells were sorted indirectly using a monoclonal antibody specific for bovine CD8 (IL-A105) followed by incubation with goat anti-mouse IgG microbeads (Miltenyi Biotec). CD14 monocytes were sorted directly by incubation with CD14 microbeads (Miltenyi Biotec). PBMC and $CD8^+$ T cells were added to wells ($2.5x10^5$/well) of coated/blocked ELISpot plates and stimulated with autologous TpM ($2.5x10^4$/well) or Tp2 peptides (1μg/ml final concentration). Purified monocytes were additionally added ($2.5x10^4$/well) to wells containing peptide and $CD8^+$ T cells. ELISpot plates were incubated and developed as described above.

In order to recall Tp2 peptide specific CTL responses, PBMC were stimulated with autologous TpM 14 days post-challenge as described above. Viable cells were harvested 7 days post-stimulation and lytic activity against TpM and Tp2 peptide pulsed autologous iSF assessed as described above.

Optimization of INF-γ ELISpot for the recognition of target antigens by schizont specific CTL.

The ability of the IFN-γ ELISpot to detect the recognition of TpM by CTL was first assessed using a $CD8^+$ polyclonal CTL line from animal F100. Fourteen days post-stimulation, CTL were added (5000/well) to coated/blocked ELISpot wells containing 25,000 irradiated autologous schizont and the formation of IFN-γ spots assessed after a 20-hour incubation (Figure 1). Pre-incubating the TpM for 30 min with a mAb against BoLA class I completely inhibited the IFN-γ response whilst mAbs against MHC class II or the irrelevant CD21 antigen had no effect (Fig 1). Significantly, there was almost no spontaneous release of IFN-γ from CTL cultured without TpM. This TpM line did not constitutively express TpM and no IFN-γ spots could be attributed to the TpM (Data not shown).

In an attempt to replicate the transient transfection situation, where CTL would be co-cultured with COS-7 or iSF of which only a small proportion of cells would be expressing the target antigen, TpM were titrated in COS-7 or iSF and co-cultured with CTL in IFN-γ ELISpot plates (Fig 2). The stimulator population was fixed at an input of 40,000/well, with only the proportions of TpM and COS-7/SF varying. This cell input was adopted since it was thought to mirror the numbers of APC that would be co-cultured with CTL after transient transfection. Initially different CTL inputs were tried against TpM titrations with the aim of identifying the minimum CTL input required to detect significant responses to 1-3% TpM (Fig 2 A). A CTL input of 10,000/well was determined to be optimal since it could elicited significant responses to less than 1% TpM and it was practically feasible to raise such CTL numbers for screening experiments.

Further titration experiments were performed with CTL clones from F100 to confirm that with these CTL and APC inputs the IFN-γ ELISpot was meeting or exceeding the desired sensitivity level (Fig 2 B). With all the clones tested the IFN-γ ELISpot could still detect recognition of target cells when they constituted only 0.1% of the total cell population.

The ELISpot assay worked well with little background noise and met the sensitivity requirements when TpM were titrated in COS-7 cells but it was important to determine that the assay performed as well when the TpM were titrated in autologous SF. Fig 2 C shows the results of co-culturing F100 $CD8^+$ polyclonal CTL line with TpM diluted in COS-7 cells, autologous primary SF and iSF. Neither primary nor iSF significantly affected the background levels or the sensitivity of the ELISpot assay.

All experiments to that date had been performed with CTL harvested 14 days post-stimulation and therefore a time course experiment was initiated to determine the optimal time post-stimulation to use CTL to detect recognition of target cells by IFN-γ ELISpot (Figure 3). CTL were harvested at various time-points from day 7 to day 20 post-stimulation and responses to TpM titrations assessed. There was no significant difference in the magnitude of responses of the CTL harvested at the times tested and therefore it was decided that CTL at any time between day 7 and 18 post-stimulation were appropriate for use in screening experiments.

In advance of the initiation of screening for CTL target antigens by the transient transfection of COS-7 cells and iSF, the efficiencies of COS-7 and iSF transfection in 96 well TC plates was assessed using GFP as a reporter gene. Whilst there was considerable variation in transfection efficiencies between cell lines and between experiments a representative result is shown in Table 5. COS-7 consistently transfected better than iSF with efficiencies varying from 5-50% whereas for iSF transfection efficiency ranged between 0.5 – 20%. The transfection efficiency of iSF was assessed to be good enough to allow the presentation and identification of transfected schizont cDNA.

The conclusions that iSF were appropriate APC and that the ELISpot had been sufficiently optimized using TpM was rapidly validated by the identification of CTL target schizont antigens initially with Tp1 and COS-7 cells and then Tp2 with iSF. The effect of reduced DNA input upon subsequent recognition of target antigen transfected COS-7 cells and iSF were compared using Tp1 and Tp2 (Figure 4). The results showed recognition of target antigen transfected COS-7 or BW014 when only 1ng/well if target antigen DNA is used to transfect. The response

Cell type	% transfected
BW013 iSF	1
BW014 iSF	7
BV050 iSF	6
COS-7	27

Table 5. Transient transfection efficiencies of iSF compared to COS-7 cells. COS-7 cells and iSF lines from animals BW013, BW014 and BV050 were transfected with pTracer plasmid (Invitrogen) and the proportion of GFP expressing cells assessed 24 hours post-transfection by flow cytometry.

increased with the concentration of target DNA peaking at peaking at 50ng/well. This result validated the use of test genes or cDNA pools at 100ng/well for transfection of both COS-7 and iSF.

Identification of Tp2 following the screening of selected genes with BW002, BW013 and BW014 CD8+ polyclonal lines and D409 CTL clone #10.

Tp2 was identified through the screening of selected gene transfected autologous iSF with CTL from BW002, BW013, BW014 and D409 (Figure 5). Selected gene #5 was specifically recognized by all CTL and was named Tp2. Tp2 transfected iSF were also specifically lysed by CTL as assessed by ^{51}Chromium release assays (Figures 6, 7, 8). MHC restriction of Tp2 recognition was assessed by the introduction of blocking anti-BoLA class I mAbs. Anti-class I mAb resulted in the inhibition of lysis. The introduction of a mAb (IL-A13) that specifically binds to the BoLA class I molecule w7 also inhibited the lysis of Tp2 transfected targets by a CTL clone from D409, suggesting that the recognition was restricted by w7.

Mapping of the Tp2 CTL epitopes.

A cleaved PepSet library of 84 peptides were synthesized to cover the full length of the Tp2 protein (Mimotopes). Recognition of Tp2 peptides was assessed by IFN-γ ELISpot using autologous iSF as antigen-presenting cells. Figure 9 shows the results of screening the Tp2 Pepset with BW002, BW013, BW014 CD8$^+$ polyclonal CTL lines and D409 CTL clone#10. The BW002 polyclonal line responded to a single peptide (#43; CSHEELKKLGML) when the peptides were used at a concentration of 1pg/ml. BW0013 CTL responded significantly to two pairs of overlapping peptides; peptides #53 and 54 (FKSSHGMGKVGKRY) and peptides #78 and 79 (FAQSLVCVLMKCRG). BW014 CTL also responded to peptides #78 and 79 suggesting a common epitope. This was not perhaps surprising since BW013 and BW014 shared a sire. The w7 restricted D409 CTL clone responded to peptides #77 and 78 (KCFAQSLVCVLMKC), suggesting an overlapping epitope. In order to define the minimal length epitope for the shared BW013 and BW014 CTL epitope, all possible 9, 10 and 11mers covering FKSSHGMGKVGKRY and FAQSLVCVLMKCRG were synthesized and screened by IFN-γ ELISpot. BW013 responded to the 11mer KSSHGMGKVGK and not to any shorter sequence suggesting this was the epitope (data not shown). The results of screening the six possible 9mers of FAQSLVCVLMKCRG are shown in Figure 10. The results revealed that both BW013 and BW014 CTL responded to only the 9mer QSLVCVLMK suggesting that this was the minimal length epitope. The peptides were also screened with D409 clone #10 and found that only the

9mer peptide FAQSLVCVL was recognized. Whether this or the 8mer FAQSLVCV is the minimal length epitope remains to be determined. ^{51}Chromium-release assays were performed with peptide pulsed autologous iSF. Figures 11 and 12 illustrate that both BW014 and D409 CTL lysed Tp2 peptide pulsed iSF with levels of lysis comparable to TpM. The lysis was very specific with almost 100% lysis of iSF pulsed with Tp2 peptide 75B (QSLVCVLMK) and background levels of lysis of iSF pulsed with Tp2 peptide 76B (SLVCVLMKC).

Kinetics of Tp2 specific $CD8^+$ T cell responses from immune cattle following challenge with *T. parva* sporozoites.

Cattle BW002, BW013 and BW014, whose CTL lines had recognized Tp2, were challenged with a lethal dose of *T. parva* (Muguga) sporozoites and the responses of PBMC and purified CD8+ T cells to TpM and Tp2 peptides were measured longitudinally. All animals were solidly resistant to challenge with none experiencing fever or detectable parasitosis (data not shown).

Figures 13, A-C show the TpM and Tp2 specific CD8+ T cell responses of BW002, BW014 and BW013. From day 9 post-challenge, CD8+ T cells responded specifically to the Tp2 peptides containing the previously identified CTL epitopes. The responses increased rapidly exceeding the response to TpM and were sustained over the period of observation (day 13 post-infection). The frequency of Tp2 epitope responding CD8+ cells peaked around 1:500 for both BW002 (A) and BW014 (B), the Tp2 specific response of BW013 was significantly weaker (C). The kinetics of this response is comparable to that previously described for schizont specific CTL in efferent lymph following challenge of immune cattle with *T. parva* sporozoites.

Attempts were made to detect Tp2 specific lytic responses directly in peripheral blood post-challenge but these failed (Data not shown). An experiment was instigated to first expand schizont specific CTL numbers by a single *in vitro* stimulation with TpM and then to assess Tp2 specific lysis. Stimulated cells exhibited extremely high cytotoxic activity against both TpM and fibroblasts pulsed with a Tp2 peptide known to contain a CTL epitope (Figure 14). These results provide the first evidence direct from immune animals that Tp2 may be involved in protection against ECF.

Tp2; Hypothetical protein, chromosome 1
Nucleotide sequence: SEQ ID NO: 1

ATGAAATTGGCCGCCAGATTAATTAGCCTTTACTTTATTATTTACATTTTACATT
CCCCAGTGCTGGGAGGTAATTGTAGTCATGAAGAACTAAAAAAAATTGGGAAT
GCTAGAGGGCGATGGTTTCGACAGGGATGCATTGTTCAAATCATCACATGGT

ATGGGAAAGGTAGGAAAAAGGTATGGTCTTAAAACTACTCCAAAAGTAGATAA
AGTCTTAGCAGATCTTGAAACACTGTTTGGAAAACACGGTCTTGGTGGTATTA
GTAAAGATTGTCTTAAATGTTTTGCACAAAGCCTAGTGTGCGTATTAATGAAAT
GTAGAGGAGCATGTCTCAAAGGACCATGTACTGACGACTGCCAAAATTGCTT
TGATAGAAACTGTAAATCTGCATTGCTGGAATGCATTGGGAAAACAAGTATTC
CAAATCCATGTAAATGGAAAGAAGATTATCTAAAATACAAATTTCCTGAAACAG
ATGAGGACGAATCTACGAAAAAAGGAGAAGCCTCCGGCACTTCATAG

Amino acid sequence (Signal peptide, 1-23): SEQ ID NO:4
MKLAARLISLYFIIYILHSPVLGGNCSHEELKKLGMLEGDGFDRDALFKSSHGMGK
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CLKGPCTDDCQNCFDRNCKSALLECIGKTSIPNPCKWKEDYLKYKFPETDEDES
TKKGEASGTS

Tp2 Epitope 1 SEQ ID NO: 7
SHEELKKLGML

Tp2 Epitope 2, SEQ ID NO: 8
KSSHGMGKVGK

Tp2 Epitope 3 SEQ ID NO: 9
FAQSLVCVL

Tp2 Epitope 4 SEQ ID NO: 10
QSLVCVLMK

Tp2 Epitope I SEQ ID NO 11
AGTCATGAAGAACTAAAAAAATTGGGAATGCTA

Tp2 Epitope 2 SEQ ID NO: 12
AAATCATCACATGGTATGGGAAAGGTAGGAAAA

Tp2 Epitope 3 SEQ ID NO: 13
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Tp2 Epitope 4 SEQ ID NO: 14
CAAAGCCTAGTGTGCGTATTAATGAAA

Tp3; Hypothetical protein, chromosome 1
Nucleotide sequence: SEQ ID NO: 2
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TAA

Amino acid sequence (Signal peptide, 1-16): SEQ ID NO: 5
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Tp6; putative prohibitin, chromosome 1
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Amino acid sequence (Signal peptide, 1-29): SEQ ID NO:6
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DIYRHLGKDYDERVLPSIINEVLKSIVAQYNASQLITQRERVSKAVRDQLVNRARD
FNILLDDVSLTHLSFSPEYEKAVEAKQVAQQQAERSKYIVLKAQEEKKSTIIKAQG
ESEAARLIGSAIKDNPAFITLRRIETAKEVANILSKSQNKIMLNSNTLLLSTDK

5. Protein Expression

5.1 Construction of Plasmids

The reading frames of *T. parva* candidate antigen genes were amplified by PCR using Taq polymerase (Promega, Madison, WI USA 53711) from the original full-length genes in their respective plasmids in pcDNA3. Both the forward and reverse primers contained restriction enzyme sites (Table 6). The PCR products were digested with the respective restriction enzymes, and ligated into bacterial His-tag expression vectors, pQE30 (Qiagen, 28159 Avenue Stanford, Valencia, CA91355) or PET28 (Novagen, Madison, WI 53719 USA). The plasmids were transformed into *E. coli* strain DH5α (Life Technologies, Carlsbad, CA 92008, USA). All the plasmids were sequenced to ensure that they harbored no substitutions compared to the original genes. Purified plasmids were then used to transform competent BL21 DE3 bacterial cells.

Table 6. Primers, restriction sites and vectors used for the cloning of *T. parva* antigens for protein production.

Gene (Plasmid)	Forward Primer (Restriction Site)	Reverse Primer (Restriction Site)	PCR Product (kb)
Tp2 (pTarget)	5'GGTAATTGTAGTCATGAAGAAC3' (*Bam*HI)	5'TTTACTAATACCACCAAGA CCGTG3' (*Sal*I)	0.8

Expression

Single BL21 DE3 bacterial colonies bearing the recombinant plasmids were isolated and cultured in 2XYT (formula) at 37^{0}C to an OD_{600} of 0.6, and protein expression induced by addition of IPTG to a final concentration of 1mM, and further cultured for 4 h. The cells were harvested by centrifugation at 4000 g for 20 min. Recombinant proteins were isolated by either the native or denaturing nickel-nitrilotriacetic (Ni-NTA) agarose according to the manufacturer's protocol (Qiagen, 28159 Avenue Stanford, Valencia, CA91355), dialyzed against PBS and stored at -20 °C. Purified proteins were checked by checked on 12% SDS-PAGE gels (Laemmli, 1970) and western blot. Protein concentration was determined by the BCA Protein Assay reagent (PIERCE, Rockford, IL 61105, USA).

Western blotting

Bacterial cells or purified proteins were applied to a 12% SDS-PAGE gel under denaturing conditions (Laemmli, 1970). Proteins were electroblotted onto nitrocellulose sheets (Schleicher and Schull, Dassel, Germany). Mouse His-tag antibody (SIGMA) was used as the primary antibody, while anti mouse horseradish peroxidase conjugate (SIGMA) was used as the secondary antibody followed detection with 3,3'-Diamnobenzidine and hydrogen peroxide.

RESULTS

The reading frame of the segments of Tp2 amplified and cloned into the bacterial expression vector harbored no substitutions compared to the original gene sequences (not shown). The recombinant protein containing His-tag was produced from the construct (Figure 15), and can be determined by immunoblotting using His-tag antibody.

Results shown in Figure 16, show the results of an: SDS-PAGE (12.5%) analysis of over-expression of recombinant Tp3 in *E. coli*. The complete ORF of Tp3 containing 5' *Bam*HI and 3' *Bgl* II was PCR amplified and cloned into plasmid vector pQE-16 (Qiagen) from which DFHR fragment was excised using *Bam*H I and *Bgl* II. The recombinant plasmid was transformed into E. coli JM109 and plated. Two colonies (Tp3-1 and Tp3-2) were picked for over-expression as described in the Methods section. Tp3-1 was expressed in LB broth (Tp3-1a) or in 2X YT (Tp3-1b) while Tp3-2 was expressed only in LB broth. An irrelevant protein of 50 kDa was used as control (TLTF). Lane MW is the size makers indicated in kiloDalton (kDa).

Results shown in Figure 17., SDS-PAGE (12.5%) analysis of purified recombinant. A partial fragment (70%) of Tp6 was PCR amplified and cloned into plasmid vector pQE-16 (Qiagen). The recombinant plasmid was transformed into *E. coli* JM109. A number of colonies were identified for over-expression. The protein has been purified using an anti-histag antibody. Size markers are indicated in kiloDalton (kDa).

OUTLINE OF THE INVENTION:

1. An isolated nucleic acid represented by SEQ ID NO: 1, SEQ ID NO: 2, or SEQ ID NO: 3, or a mutant, variant, complement, or fragment thereof.

1a. The nucleic acid of claim 1, which is represented by SEQ ID NO 1.

1b. The nucleic acid of claim 1, which is represented by SEQ ID NO: 2.

1c. The nucleic acid of claim 1, which is represented by SEQ ID NO: 3.

2. The isolated nucleic acid of claim 1, wherein the mutant reflects the degeneracy of the genetic code.

3. The isolated nucleic acid of claim 1, wherein the variant is a nucleic acid that is at least about 80% identical to a nucleic acid represented by SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3.

4. The nucleic acid of claim 1, wherein the variant is a nucleic acid that is at least about 95% identical to a nucleic acid represented by SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3.

5. The nucleic acid of claim 1, wherein the variant is a nucleic acid that is at least about 98% identical to a nucleic acid represented by SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3.

6. The nucleic acid of claim 1, wherein the variant hybridizes specifically under stringent conditions to a nucleic acid represented by SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3.

7. An isolated nucleic acid represented by SEQ ID NO: 11, 12, 13, 14, or a mutant, variant, complement or fragment thereof.

7a. The nucleic acid of claim 7, which is represented by SEQ ID NO: 11.

7b. The nucleic acid of claim 7, which is represented by SEQ ID NO: 12.

7c. The nucleic acid of claim 7, which is represented by SEQ ID NO: 13.

7d. The nucleic acid of claim 7, which is represented by SEQ ID NO: 14.

8. An isolated nucleic acid which encodes a polypeptide represented by SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6.

9. An isolated polypeptide represented by SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6, or a mutant, variant or fragment thereof.

9a. The polypeptide of claim 9, which is represented by SEQ ID NO 4.

9b. The polypeptide of claim 9, which is represented by SEQ ID NO 5.

9c. The polypeptide of claim 9, which is represented by SEQ ID NO 6.

10. The isolated polypeptide of claim 9, wherein the variant is a polypeptide that is at least about 80% identical to a polypeptide represented by SEQ ID NO:4, SEQ ID NO:5, or SEQ ID NO:6.

11. The isolated polypeptide of claim 9, wherein the variant is a polypeptide that is at least about 95% identical to a polypeptide represented by SEQ ID NO:4, SEQ ID NO:5, or SEQ ID NO:6.

12. The isolated polypeptide of claim 9, wherein the variant is a polypeptide that is at least about 98% identical to a polypeptide represented by SEQ ID NO:4, SEQ ID NO:5, or SEQ ID NO:6.

13. An isolated polypeptide represented by SEQ ID NO: 7, 8, 9, 10, or a mutant, fragment or variant thereof.

13a. The polypeptide of claim 13, which is represented by SEQ ID NO 7.

13b. polypeptide of claim 13, which is represented by SEQ ID NO8..

13c. polypeptide of claim 13, which is represented by SEQ ID NO 9.

13d. polypeptide of claim 13, which is represented by SEQ ID NO 10.

14. The polypeptide of claim 9 or 13, which functions as a stimulant to cytotoxic T cells (CTL).

15. A vector comprising a nucleic acid of claim 1, claim 7 or claim 8.

16. The vector of claim 15, wherein said nucleic acid is operatively linked to an expression control sequence.

17. The vector of claim 15, further comprising a sequence encoding a selectable marker polypeptide.

18. A host cell comprising a vector of claim 16 or claim 17.

19. A method for producing a polypeptide represented by SEQ ID NO: 4, 5, 6 or 8, comprising culturing a cell of claim 18 under conditions effective for the cell to express the polypeptide, and harvesting the polypeptide.

20. An immunogenic composition, comprising a polypeptide of claim 9 and/or claim 13.

21. The immunogenic composition of claim 20, further comprising an adjuvant.

22. The immunogenic composition of claim 20 or 21, which is a vaccine.

23. A pharmaceutical composition, comprising a polypeptide of claim 9 and/or claim 13 and a pharmaceutically acceptable carrier.

24. A kit for detecting the presence of a protozoan organism in a sample suspected of containing the organism, comprising a detectably labeled nucleic acid of claim 1, claim 7 and/or claim 8, and, optionally, means for detecting the detectably labeled nucleic acid.

24a. The kit of claim 24, wherein the protozoan organism is a Theileria.

24b. The kit of claim 24a, wherein the Theileria is T. parva.

25. A kit for detecting the presence of a protozoan organism in a sample suspected of containing the organism, comprising a polypeptide of claim 9 and/or claim 13, and, optionally, means for detecting the polypeptide.

25a. The kit of claim 25, wherein the protozoan organism is a Theileria.

25b. The kit of claim 25a, wherein the Theileria is T. parva.

26. An antibody specific for a polypeptide of claim 9 or claim 13.

26a. The antibody of claim 26, which is a monoclonal antibody.

26b. The antibody of claim 26, which is a polyclonal antibody.

26c. A kit for detecting the presence of a protozoan organism in a sample suspected of containing the organism, comprising an antibody of claim 26, and, optionally, means for detecting the antibody.

26d. The kit of claim 26c, wherein the protozoan organism is a Theileria.

26e. The kit of claim 26d, wherein the Theileria is T. parva.

27. A method for detecting a protozoan organism in a sample suspected of containing the organism, comprising contacting the sample with a detectably labeled nucleic acid of claim 1, claim 7 and/or claim 8, and detecting detectably labeled nucleic bound to a nucleic acid of the organism.

27a. The method of claim 27, wherein the protozoan organism is a Theileria.

27b. The method of claim 27a, wherein the Theileria is T. parva.

28. A method for detecting a protozoan organism in a sample suspected of containing the organism, comprising contacting the sample with a polypeptide of claim 9 and/or claim 13, and detecting the polypeptide bound to an antibody specific for the organism which is in the sample.

28a. The method of claim 28, wherein the protozoan organism is a Theileria.

28b. The method of claim 28a, wherein the Theileria is T. parva.

29. A method for detecting a protozoan organism in a sample suspected of containing the organism, comprising contacting the sample with an antibody of claim 26, and detecting the antibody bound to a protein from the organism which is in the sample.

29a. The method of claim 29, wherein the protozoan organism is a Theileria.

29b. The method of claim 29a, wherein the Theileria is T. parva.

30. A method for eliciting an immunogenic response in an animal comprising administering to the animal an effective amount of an immunogenic composition of claim 20 or claim 21.

31. A method for vaccinating an animal against infection by a protozoan organism, comprising administering to the animal an effective amount of a vaccine of claim 22.

31a. The method of claim 31, wherein the protozoan organism is a Theileria.

31b. The kit of claim 31a, wherein the Theileria is T. parva.

32. The method of claim 31, wherein the vaccine is administered by intramuscular injection.

33. The method of claim 31, wherein the vaccine is administered by subcutaneous injection.

34. The method of claim 31, wherein the vaccine is administered by intradermal injection.

35. A vaccine of claim 22, which is suitable for intramuscular injection.

36. A vaccine of claim 22, which is suitable for subcutaneous injection.

37. A vaccine of claim 22, which is suitable for intradermal injection

38. A composition comprising a polypeptide of claim 9 and/or claim 13, or an antibody thereto, wherein the composition is an anti-cancer composition.

39. A method for identifying an anti-neoplastic agent, comprising identifying an agent that inhibits the ability of a polypeptide of claim 9 and/or claim 13 to stimulate cell proliferation, or to initiate or maintain transformation of a cell.

ABSTRACT:

This invention relates to DNA, protein, and peptide compositions and methods for identification of intracellular antigens of pathogens that stimulate animal cytotoxic lymphocytes in an antigen specific manner. Such antigens are prime candidates for the development of vaccines useful for the prevention of intracellular pathogenic diseases such as East Coast Fever. The compositions are also useful for diagnostics, and for therapy for proliferative cell diseases.

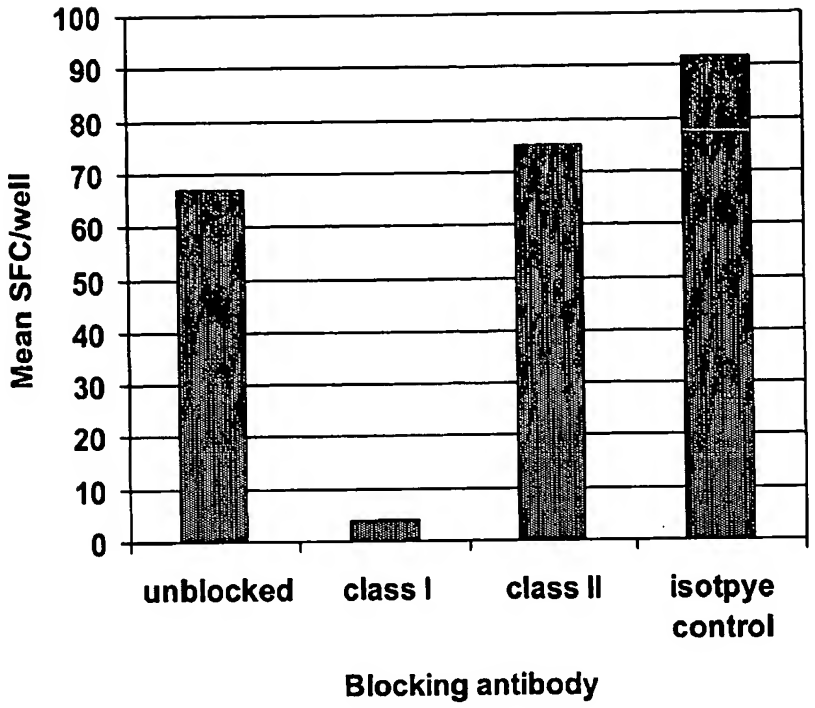
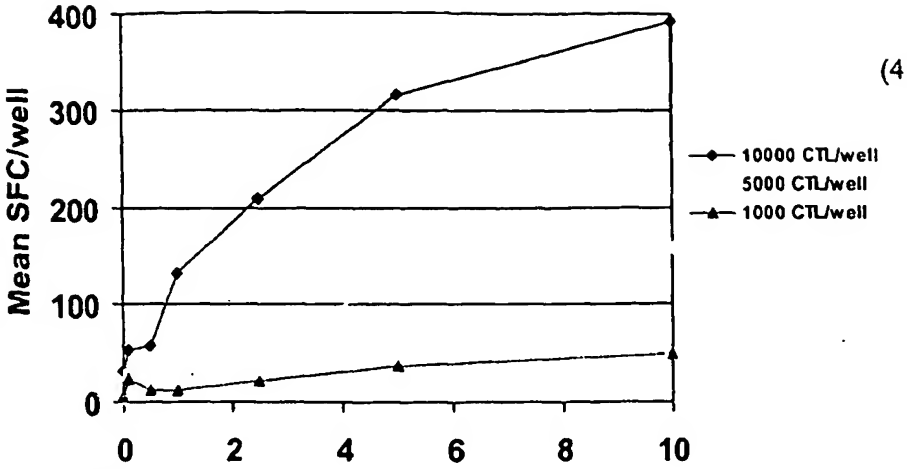


Figure 1. IFN-γ ELISpot detection of BoLA class I restricted CTL responses to schizont infected cells. Autologous TpM were pre-incubated in ELISpot plates with monoclonal antibodies specific for BoLA class I, BoLA class II or bovine CD21 (isotype control) before the addition of schizont specific polyclonal CTL. Cells were co-cultured for 20 hours before the plates were developed. IFN-γ production is presented as mean number of spot forming cells (SFC)/well.

A



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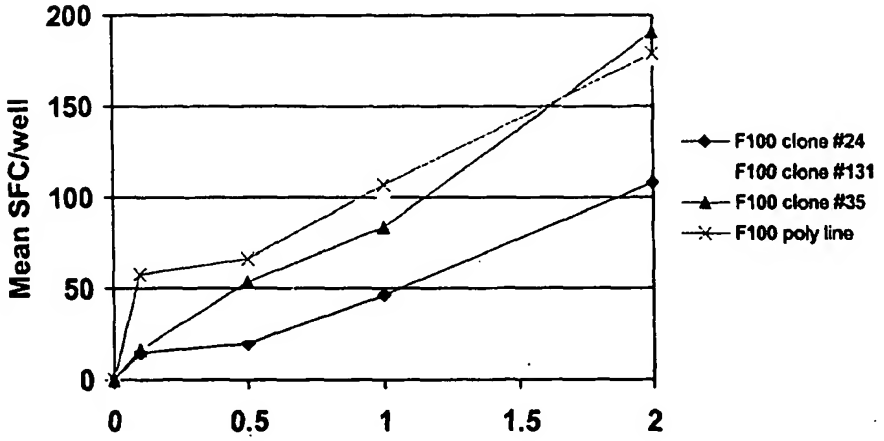

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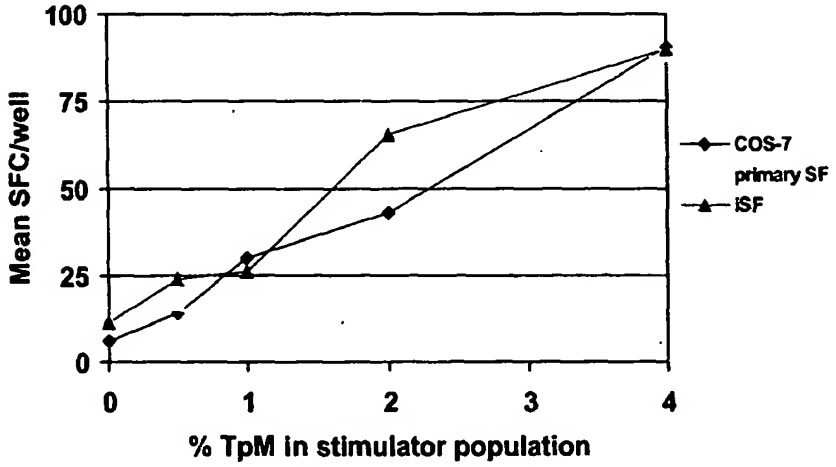

Figure 2. Sensitivity of IFN-γ ELISpot to detect CTL recognition of schizont infected cells. The sensitivity of the ELISpot was assessed by co-culturing CTL with a population of COS-7 cells or iSF into which TpM had been titrated. Initially the effects of varying the CTL input on responses to TpM titrated in COS-7 cells was addressed (A). Using a CTL input of 10,000/well the ability of the ELISpot to detect responses to very small numbers of TpM was determined (B). The effect of titrating TpM in COS-7 cells, autologous primary SF or iSF was examined and found to have no effect on the background or sensitivity of the ELISpot assay (C).

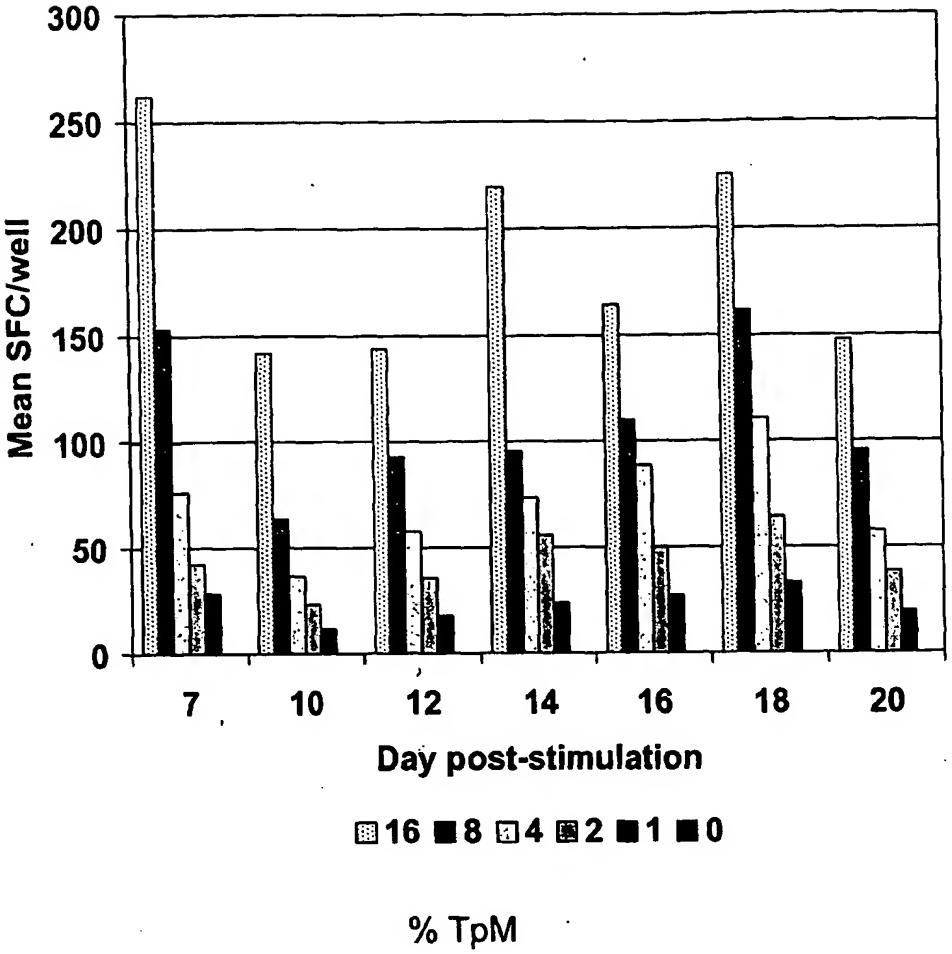

Figure 3. Effect of CTL age post-stimulation upon responses to schizont infected cells as detected by IFN-γ ELISpot. A schizont specific polyclonal CTL line was harvested at various time points after *in vitro* stimulation and the ability of the IFN-γ ELISpot to detect antigen recognition was determined with TpM titrations. IFN-γ production is presented as the mean number of spot forming cells (SFC)/well.

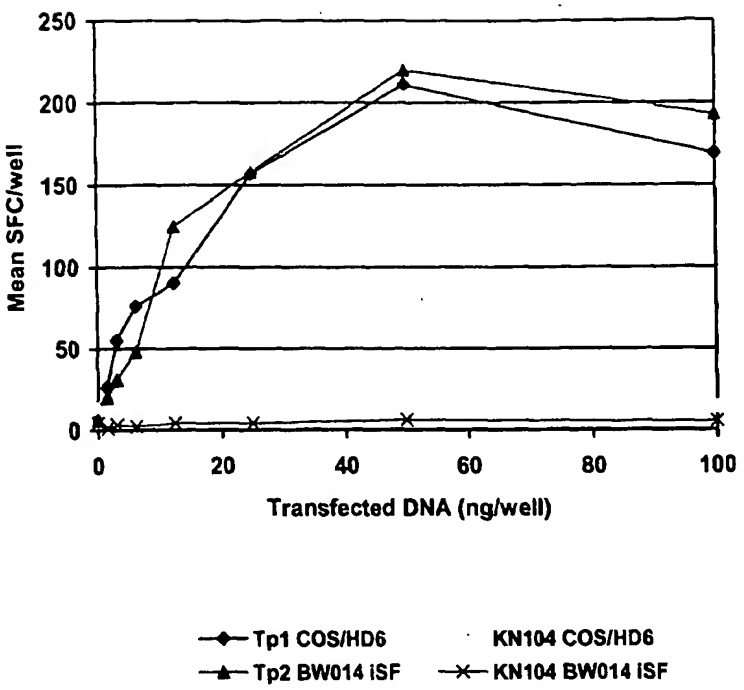

Figure 4. Effect of reduced Tp1 and Tp2 DNA for transfection of COS and iSF upon recognition by schizont specific BV115 and BW014 polyclonal CD8+ CTL lines, respectively. The total amount of DNA was kept constant (100ng/well) by titration of Tp1/Tp2 DNA in irrelevant DNA (KN104 cDNA). Recognition of transfected cells was measured using the IFN-γ ELISpot assay and responses presented as the mean number of spot forming cells (SFC)/well.

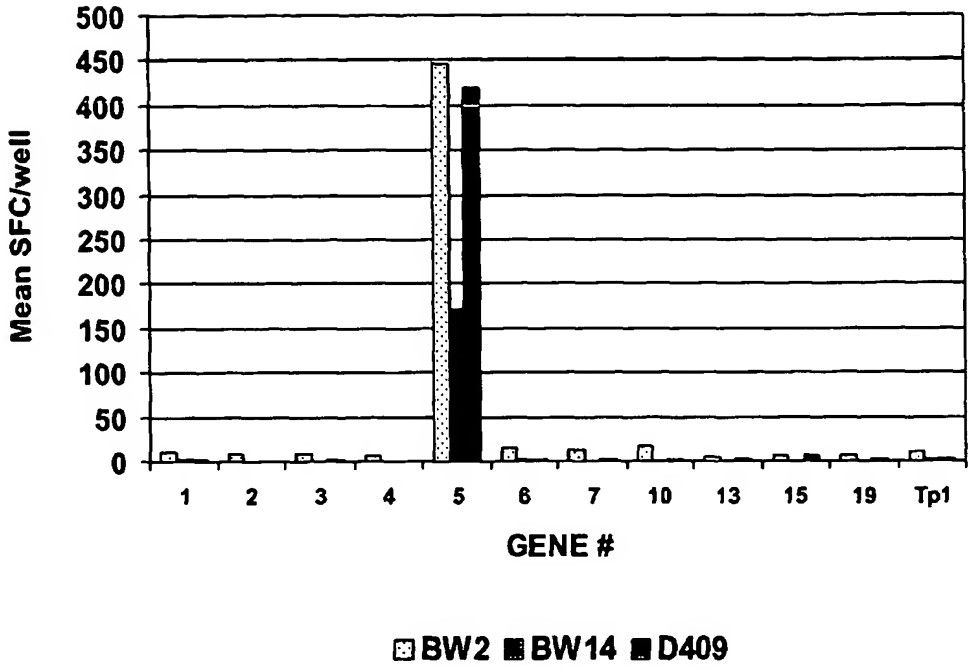

Figure 5. Recognition of selected gene #5 transfected iSF by BW002, BW014 CD8+ polyclonal CTL lines and D409 CTL clone #10. iSF transfected with selected genes and Tp1 were cultured with CTL and recognition assessed by IFN-γ ELISpot. Responses are presented as mean numbers of spot forming cells (SFC)/well.

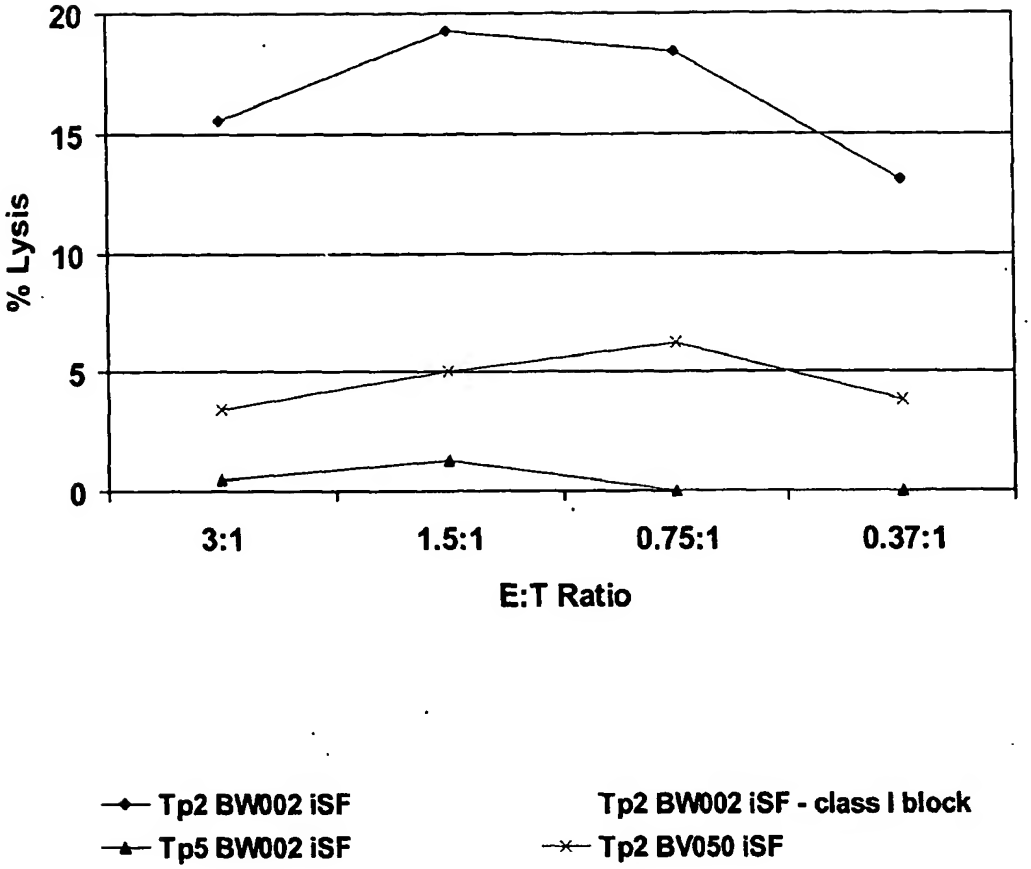

Figure 6. Lysis of Tp2 transfected autologous (BW002) and allogeneic (BV050) iSF by the schizont specific BW002 polyclonal CD8+ CTL line. Tp5 transfected autologous iSF also served as a negative control. BoLA class I restriction was assessed by pre-incubating Tp2 transfected iSF with an anti-BoLA class I mAb (class I block).

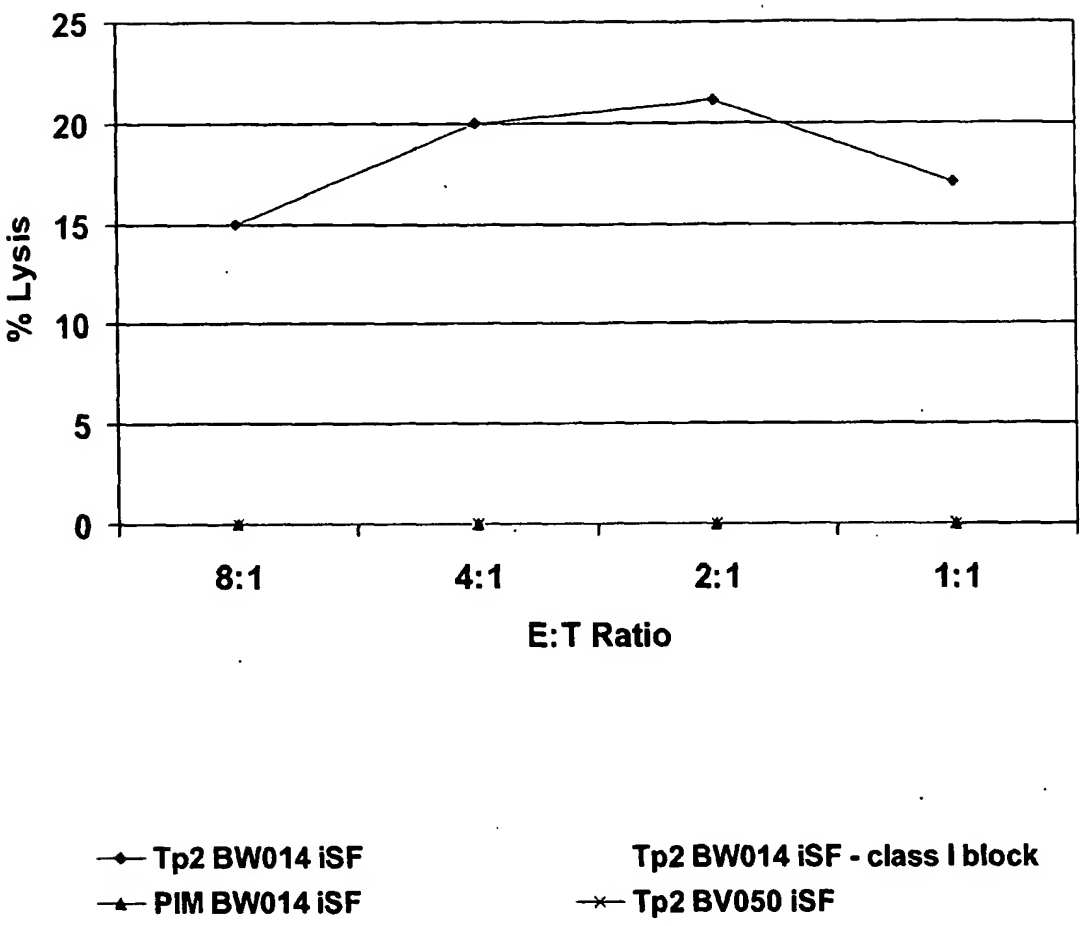

Figure 7. Lysis of Tp2 transfected autologous (BW014) and allogeneic (BV050) iSF by the schizont specific BW014 polyclonal CD8+ CTL line. PIM transfected autologous iSF also served as a negative control. BoLA class I restriction was assessed by pre-incubating Tp2 transfected iSF with an anti-BoLA class I mAb (class I block).

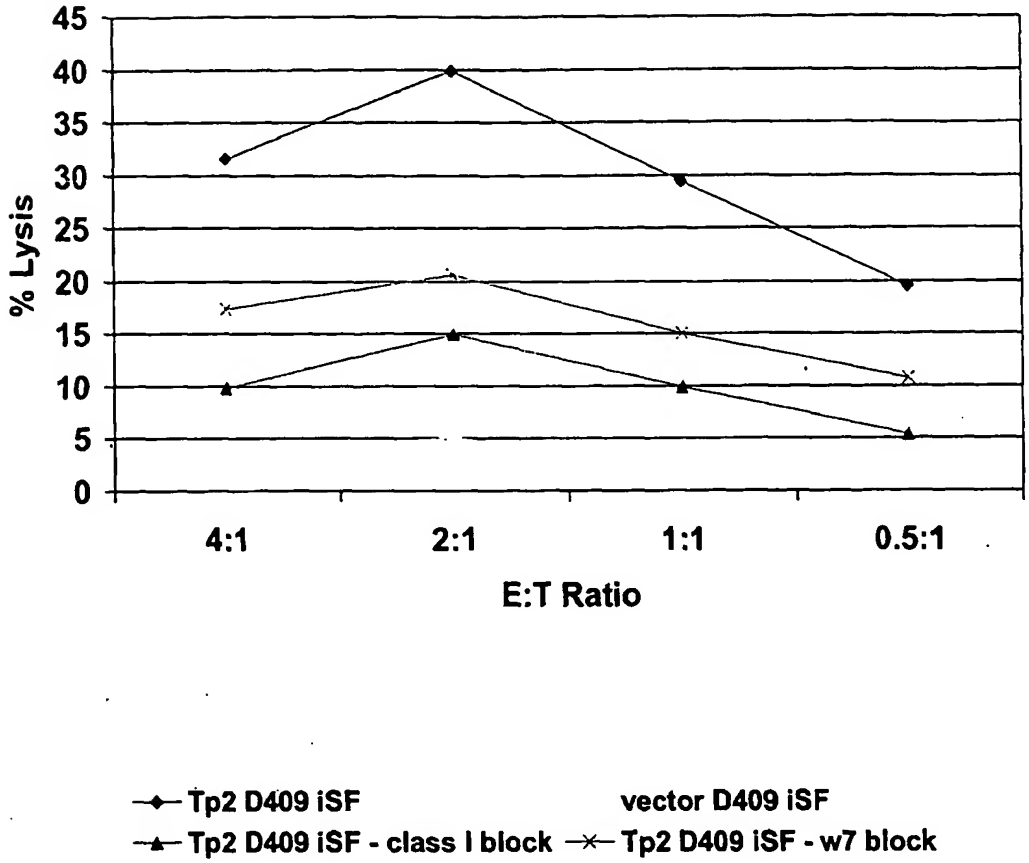

Figure 8. Lysis of Tp2 transfected D409 iSF by w7 restricted schizont specific D409 CTL clone #10. Empty plasmid vector transfected autologous iSF served as a negative control. BoLA class I restriction was assessed by pre-incubating Tp2 transfected iSF with an anti-BoLA class I mAb (class I block) and anti-w7 mAb (w7 block).

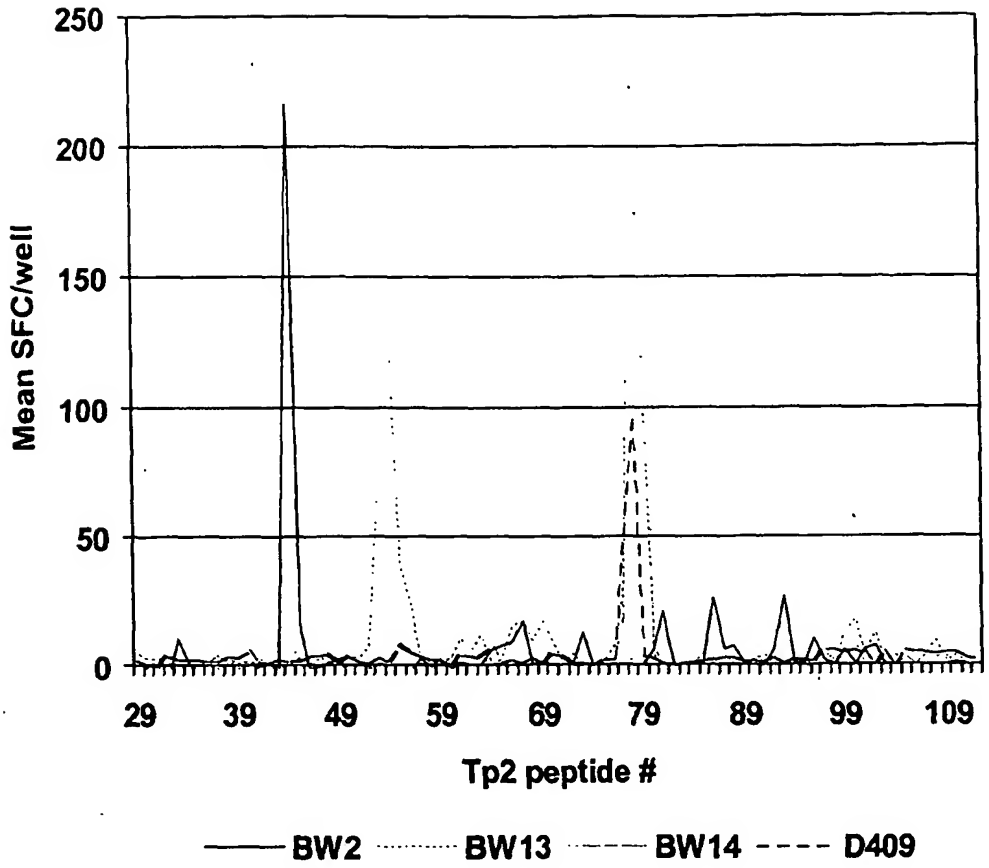

Figure 9. Mapping of Tp2 CTL epitopes using synthetic peptides. Eighty four 12mer peptides overlapping by two amino acids encompassing the full length of Tp2 were synthesised and used at a final concentration of 1pg/ml (BW002) or 1μg/ml (BW013, BW014 & D409) to pulse autologous iSF. Recognition of peptide pulsed iSF by CD8+ polyclonal lines from BW002, BW013 and BW014, and D409 CTL clone #10 was assessed by IFN-γ ELISpot. Significant responses were observed against different sets of overlapping peptides suggesting a number of unique, overlapping and shared CTL epitopes.

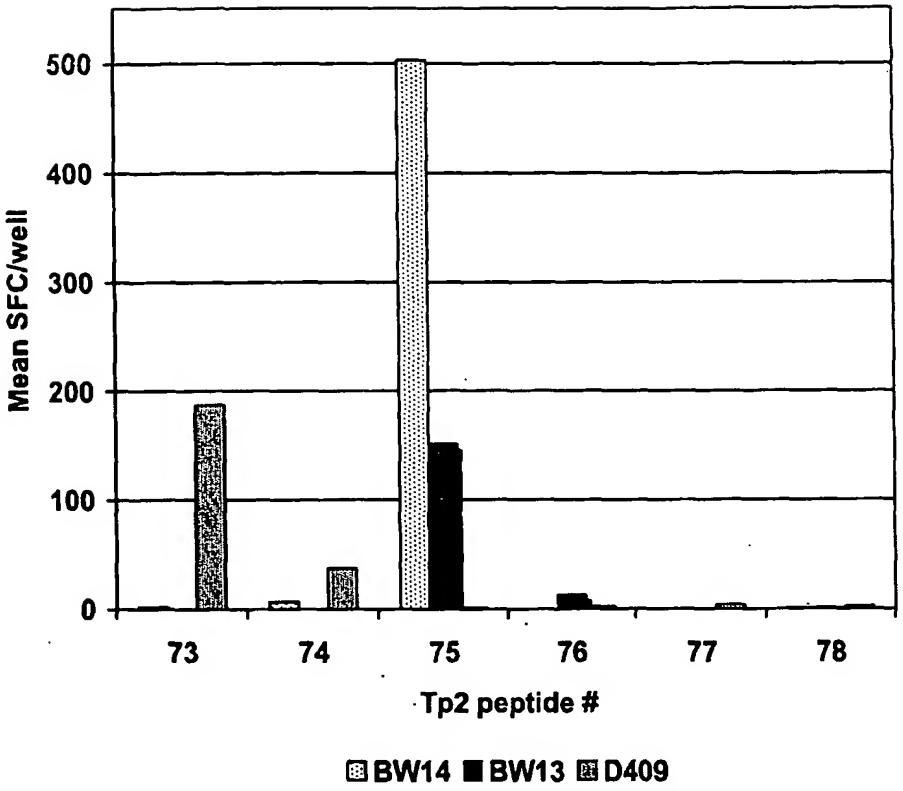

Figure 10. Identification of Tp2 CTL epitopes using synthetic 9mer peptides. The 6 possible 9mer peptides derived from the epitope containing sequence FAQSLVCVLMKCRG were synthesised and used at a final concentration of 1μg/ml to pulse autologous iSF. Recognition of peptide pulsed iSF by CD8+ polyclonal lines from BW013 and BW014, and D409 CTL clone #10 was assessed by IFN-γ ELISpot.

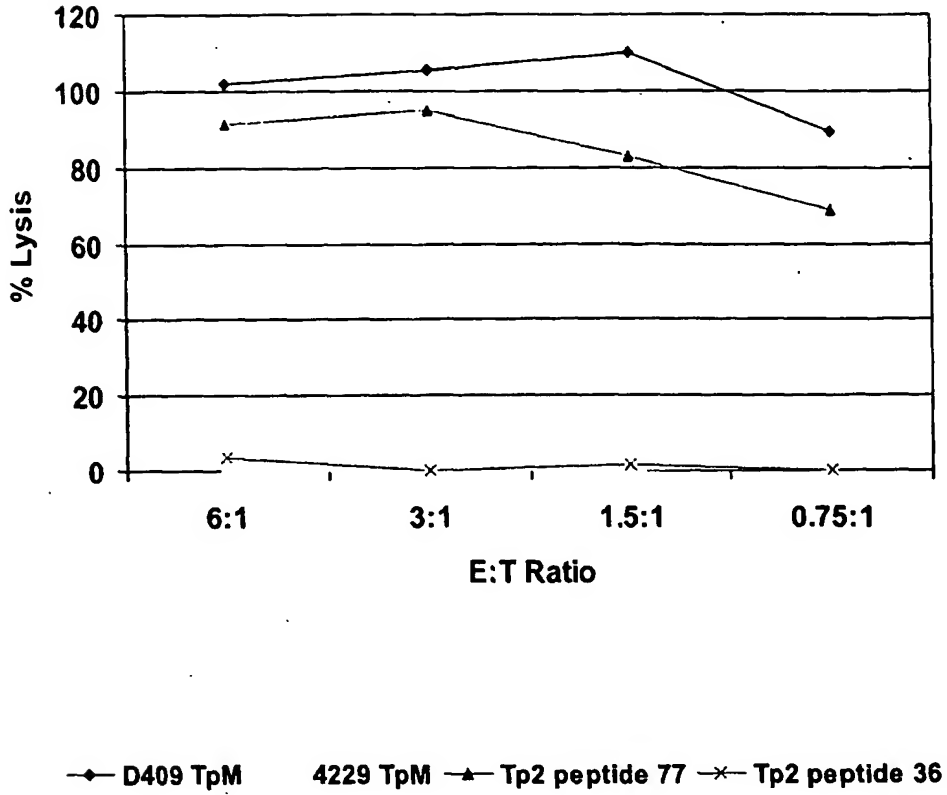

Figure 11. Lysis of Tp2 synthetic peptide pulsed autologous iSF by the schizont specific D409 CTL clone #10. D409 iSF were pulsed overnight with the Tp2 epitope containing 12mer peptide (#77; KCFAQSLVCVLM) or a control Tp2 12mer peptide (#36; FIIYILHSPVLG) at final peptide concentration of 1μg/ml. Autologous and allogeneic (4229) TpM were included as a positive and negative controls.

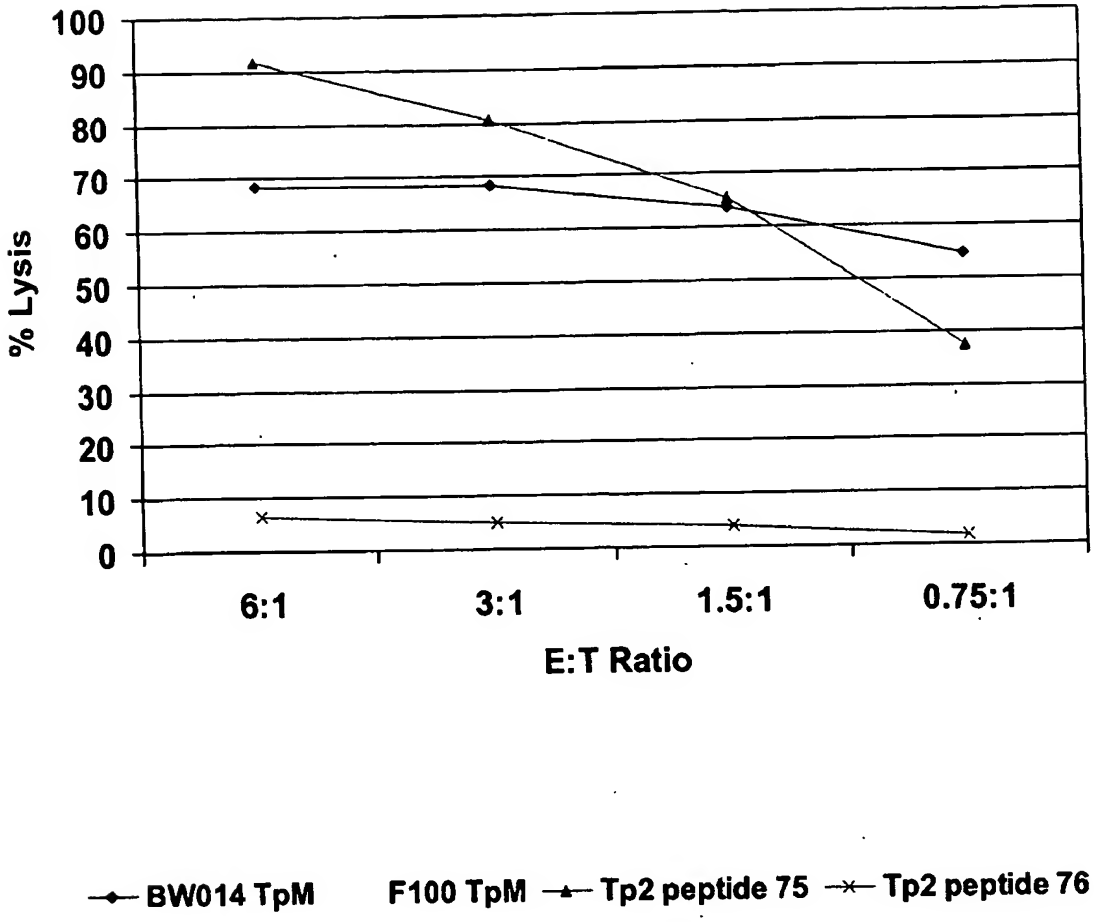

Figure 12. Lysis of Tp2 synthetic peptide pulsed autologous iSF by the schizont specific BW014 polyclonal CD8+ CTL line. BW014 iSF were pulsed overnight with the Tp2 epitope 9mer peptide (#75; QSLVCVLMK) or a control Tp2 9mer peptide (#76; SLVCVLMKC) at final peptide concentration of 1μg/ml. Autologous and allogeneic (F100) TpM were included as a positive and negative controls.

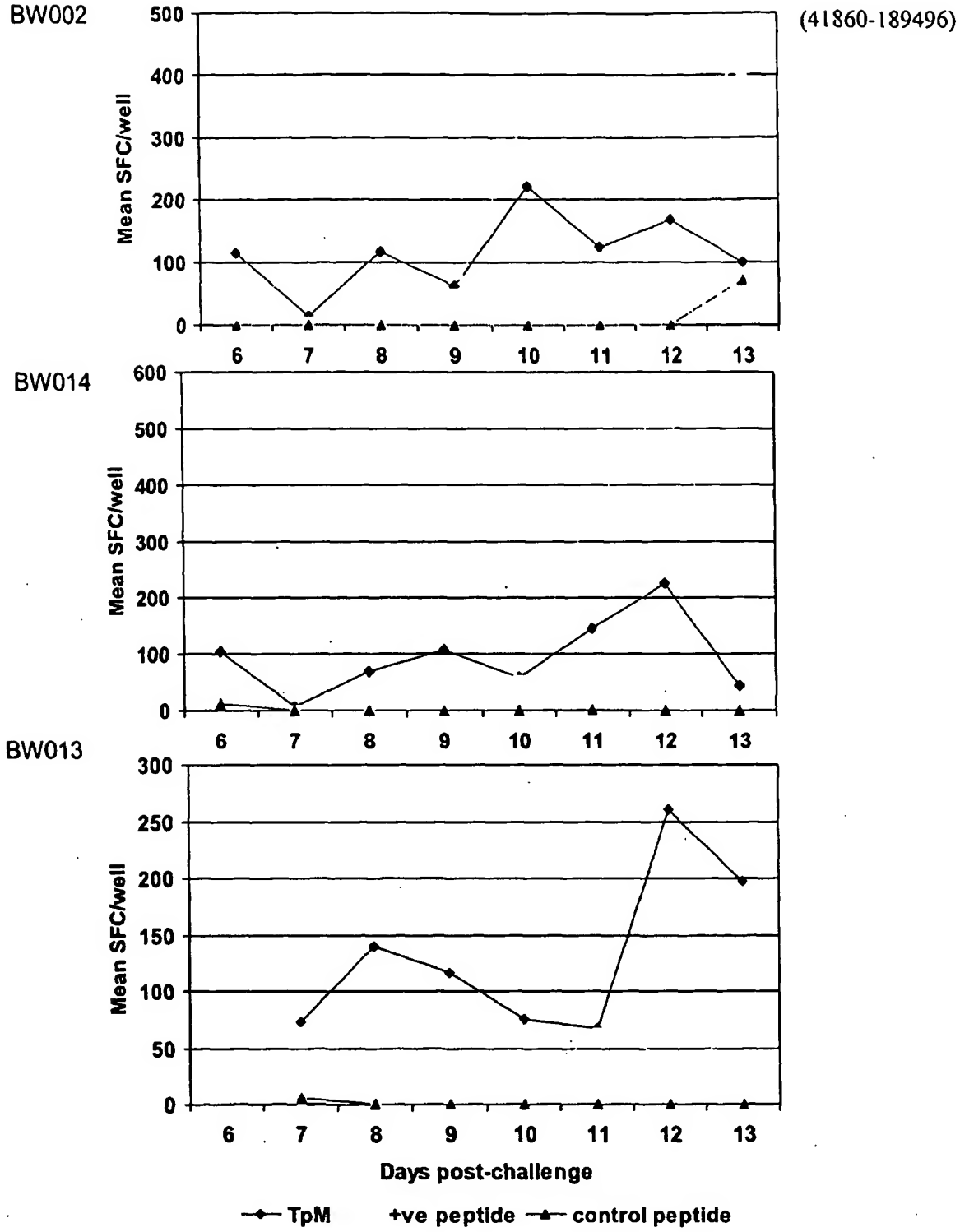

Figure 13. Tp2 specific $CD8^+$ T cell responses following challenge of immune cattle. Three ECF immune cattle (BW002, BW013, BW014), from which schizont specific CTL lines had been generated and shown to recognise Tp2, were challenged with a lethal dose of *T. parva* (Muguga) sporozoites. Peripheral blood was collected daily between day 7 – 13 post-challenge, and CD8+ T cells and monocytes purified. Responses to Tp2 peptides containing previously identified CTL epitopes or control peptides were assessed by co-culturing CD8+ T cells and monocytes in the presence of 1μg/ml peptide and measuring the release of IFN-γ by ELISpot. Responses to autologous TpM were included as a positive control

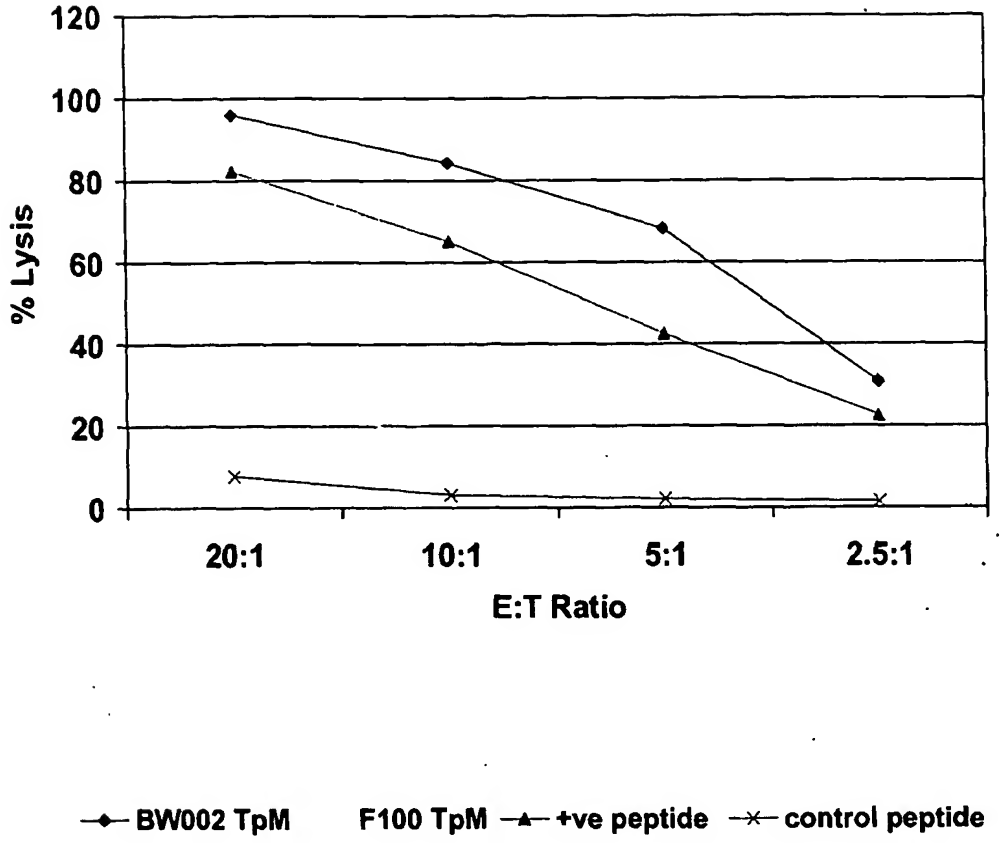

Figure 14. Lysis of Tp2 peptide pulsed autologous iSF by TpM stimulated PBMC. BW002 PBMC were collected 14 days post-challenge and co-cultured for 7 days with autologous TpM. Cells were tested for their ability to lyse Tp2 peptide pulsed autologous iSF using a ^{51}Chromium release assay. iSF were pulsed overnight with a Tp2 12mer peptide known to contain a CTL epitope (#43; +ve peptide) or a control Tp2 12mer peptide (#40; control peptide) at a concentration of 1μg/ml. Autologous and allogeneic (F100) TpM were included as positive and negative controls.

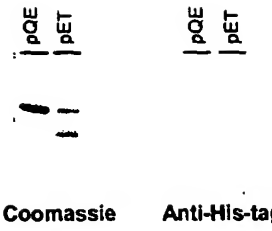

Figure 15. Expression of *T. parva* Tp2 antigen protein. Recombinant protein was isolated by Ni-NTA agarose and ran on 12% SDS-PAGE gels followed by staining using coomassie blue or detection by western blotting.

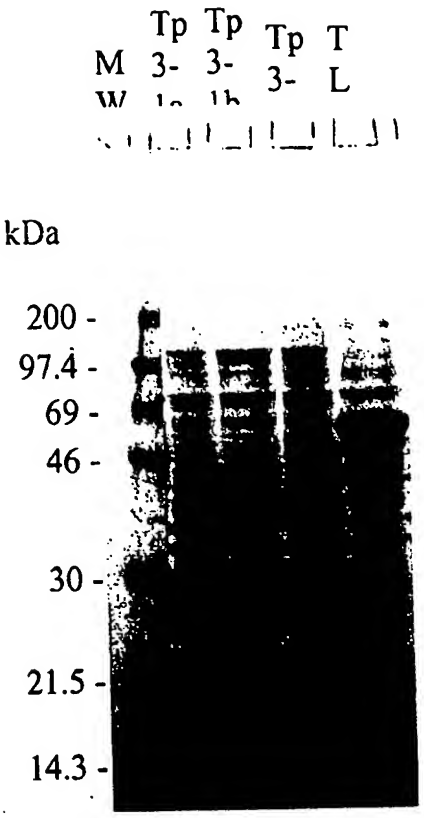

Figure 16: SDS-PAGE (12.5%) analysis of over-expression of recombinant Tp3 in *E. coli*.

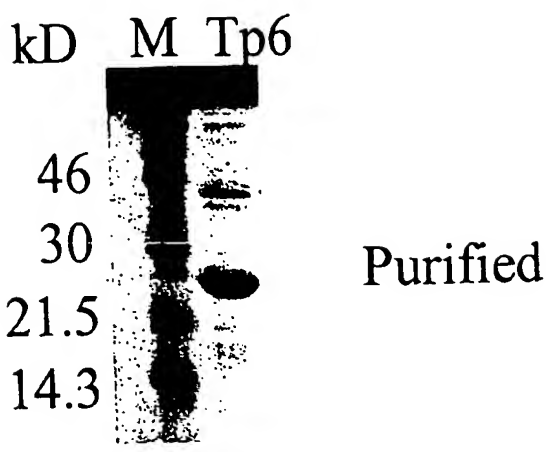

Figure 17: SDS-PAGE (12.5%) analysis of purified recombinant Tp6.

Figure 18.

Tp2; Hypothetical protein, chromosome 1
Nucleotide sequence: SEQ ID NO: 1
ATGAAATTGGCCGCCAGATTAATTAGCCTTTACTTTATTATTTACATTTTACAT
TCCCCAGTGCTGGGAGGTAATTGTAGTCATGAAGAACTAAAAAAATTGGGAA
TGCTAGAGGGCGATGGTTTCGACAGGGATGCATTGTTCAAATCATCACATG
GTATGGGAAAGGTAGGAAAAAGGTATGGTCTTAAAACTACTCCAAAAGTAGA
TAAAGTCTTAGCAGATCTTGAAACACTGTTTGGAAAACACGGTCTTGGTGGT
ATTAGTAAAGATTGTCTTAAATGTTTTGCACAAAGCCTAGTGTGCGTATTAAT
GAAATGTAGAGGAGCATGTCTCAAAGGACCATGTACTGACGACTGCCAAAA
TTGCTTTGATAGAAACTGTAAATCTGCATTGCTGGAATGCATTGGGAAAACA
AGTATTCCAAATCCATGTAAATGGAAAGAAGATTATCTAAAATACAAATTTCC
TGAAACAGATGAGGACGAATCTACGAAAAAAGGAGAAGCCTCCGGCACTTC
ATAG

Amino acid sequence (Signal peptide, 1-23): SEQ ID NO:4
MKLAARLISLYFIIYILHSPVLGGNCSHEELKKLGMLEGDGFDRDALFKSSHGMG
KVGKRYGLKTTPKVDKVLADLETLFGKHGLGGISKDCLKCFAQSLVCVLMKCR
GACLKGPCTDDCQNCFDRNCKSALLECIGKTSIPNPCKWKEDYLKYKFPETDE
DESTKKGEASGTS

Figure 19.

Tp3; Hypothetical protein, chromosome 1
Nucleotide sequence: SEQ ID NO: 2
ATGAAATTAAATACTATCGCAATAGCCTTTTTGTATTCCTGTTTCTCACAGTTT
TTAAAAAATGTGTCTGCTCTGAGGCGTAGTTCTCCAGATTTGTCACCAGATG
GTTCTTTTCTTCAAGTAAAATCAGCTTCTCCTCAGGATAAACAAGATGTAATC
CAAAGTTCCTCTCCTAAGGTAACAGTGCCTACGGTTGACCCTGAAGGCCTC
AAGAAGGCGGTTACTGCAGCAGTTCTATCAAACCAAAATCAAGCTCTACAAA
ACGGTGCTCTTAATCCAGCAGATTTCACTCAAGCTGCCTCTGTTAATTCCAT
GAGTAATGCTGTTAGTGCCATGAACAATACTGTTGGTCCAGTAAAAAATCCC
ATGGCTACTGTTGGTACTATGAACTCCTTTACTGGAATGCCTGGTGTACAGG
ATAATTTTCCTCAGACACCGCCTGTTAATGTTCAAGACACCTCTACCCAGGA
GAACAGTCTTGACAACCTAAATCTCCTCTTAGATCCCTCGTTAGTAAAGATAT
CTCAAGCTGATAGTCACATAAAAGAAAGCATGGAAAAAGCTGTACACAGCCT
TAAAAAGGTCTTGGAGGGGCTAACCAACCTTGCGACTCTGTCTAAAAGTAG
GGATACTGAACCGTTTAATGTTCTGGGGGATGACTATACGATGCGTAACGTT
TTGGACCTCATGAATAAGGAACTCAGGCAGGTTGAATCTCTTCAGAAAGTTG
TGTTCCAATTCAACGCCTTTGCACTTTCCACCTTCACTAAGAGTCCAGACGA
TAATAAAAAATCCTAA

Amino acid sequence (Signal peptide, 1-16): SEQ ID NO: 5
MKLNTIAIAFLYSCFSQFLKNVSALRRSSPDLSPDGSFLQVKSASPQDKQDVIQS
SSPKVTVPTVDPEGLKKAVTAAVLSNQNQALQNGALNPADFTQAASVNSMSNA
VSAMNNTVGPVKNPMATVGTMNSFTGMPGVQDNFPQTPPVNVQDTSTQENS
LDNLNLLLDPSLVKISQADSHIKESMEKAVHSLKKVLEGLTNLATLSKSRDTEPF
NVLGDDYTMRNVLDLMNKELRQVESLQKVVFQFNAFALSTFTKSPDDNKKS

Figure 20

Tp6; putative prohibitin, chromosome 1
Nucleotide sequence: SEQ ID NO: 3
ATGGCTCAGATTCCTGTTGATAAATTCGCTAAATTAGTTACTGGAGCCGGCT
CTGCTCTCTTATTATTCGGTTCAGGTGCCTGGCTTGTCAATTCCAGTTTATAC
GATGTTGGAGCTGGGCATAGAGCTGTTGTATATAACCGTATCACTGGAATAA
GTGAGACTACACATGGAGAAGGAACGCACTTCATAATTCCCTGGCTAGAAC
GTCCAATAATTTACGATGTGAGGACTCGTCCTAGGACTCTGATGTCTCTCAC
CGGAAGCCGTGACTTGCAGATGGTTAACATCACCTGCCGTGTGTTGTCTCG
TCCCGATGAGCGCAGACTCAGGGATATTTACAGGCACTTGGGCAAAGATTA
CGACGAGCGAGTCCTGCCTTCAATAATAAACGAGGTTCTGAAGAGTATTGT
GGCCCAGTACAACGCCTCTCAGCTCATTACTCAGAGAGAAAGAGTTAGCAA
AGCAGTCAGGGACCAGCTGGTGAACAGGGCCAGGGACTTTAATATTCTTCT
CGATGATGTCTCCTTAACCCACTTAAGCTTCAGTCCTGAATATGAAAAGGCT
GTAGAGGCTAAACAAGTAGCTCAACAGCAAGCTGAACGCAGTAAATATATA
GTGTTGAAGGCTCAGGAGGAGAAGAAATCGACGATAATTAAGGCTCAGGGA
GAGTCTGAGGCTGCAAGGCTTATTGGAAGTGCAATTAAGGATAACCCTGCC
TTTATTACGCTTCGGAGAATTGAAACCGCTAAGGAAGTGGCTAACATTCTCT
CCAAATCGCAGAATAAAATCATGCTCAATAGTAATACTCTCTTACTCTCAACT
GATAAATAA

Amino acid sequence (Signal peptide, 1-29): SEQ ID NO:6
MAQIPVDKFAKLVTGAGSALLLFGSGAWLVNSSLYDVGAGHRAVVYNRITGISE
TTHGEGTHFIIPWLERPIIYDVRTRPRTLMSLTGSRDLQMVNITCRVLSRPDERR
LRDIYRHLGKDYDERVLPSIINEVLKSIVAQYNASQLITQRERVSKAVRDQLVNR
ARDFNILLDDVSLTHLSFSPEYEKAVEAKQVAQQQAERSKYIVLKAQEEKKSTIIK
AQGESEAARLIGSAIKDNPAFITLRRIETAKEVANILSKSQNKIMLNSNTLLLSTDK

Figure 21.

Tp2 Epitope 1 SEQ ID NO: 7
SHEELKKLGML

Tp2 Epitope 2, SEQ ID NO: 8
KSSHGMGKVGK

Tp2 Epitope 3 SEQ ID NO: 9
FAQSLVCVL

Tp2 Epitope 4 SEQ ID NO: 10
QSLVCVLMK

Tp2 Epitope I SEQ ID NO 11
AGTCATGAAGAACTAAAAAAATTGGGAATGCTA

Tp2 Epitope 2 SEQ ID NO: 12
AAATCATCACATGGTATGGGAAAGGTAGGAAAA

Tp2 Epitope 3 SEQ ID NO: 13
TTTGCACAAAGCCTAGTGTGCGTATTA

Tp2 Epitope 4 SEQ ID NO: 14
CAAAGCCTAGTGTGCGTATTAATGAAA

APPLICATION DATA SHEET

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Secrecy Order in Parent Appl.::	

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Representative Customer Number:: 26694

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	Continuation of		
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